Quorum Sensing by the Bacterial Signal Autoinducer-2: Phylogenetic Distribution of the Synthesis Gene LuxS, and its Role in *Shewanella oneidensis*

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades einer

Doktorin der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

von Ágnes Mária Bodor
aus Kecskemét, Ungarn

1. Referentin: Professor Dr. Irene Wagner-Döbler
2. Referent: Professor Dr. Dieter Jahn
eingereicht am: 04.06.2008
mündliche Prüfung (Disputation) am: 18.07.2008

Druckjahr 2008

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch die Mentorin/den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Publikationen

Bodor, A., Elxnat, B., Thiel, V., Schulz, S. & Wagner-Dobler, I. (2008). Potential for *luxS* related signalling in marine bacteria and production of autoinducer-2 in the genus *Shewanella*. *BMC Microbiol* **8**, 13.

Tagungsbeiträge

Bodor, A., Schulz, S. & Wagner-Dobler, I.: Phylogenetic distribution of the *luxS* and *sahH* genes in marine bacteria and production of autoinducer-2 in the genus *Shewanella*. (Poster) 336. 11th International Symposium on Microbial Ecology, August 20-25, Vienna, Austria (2006).

Contents

Index of tables

Index of figures

Abbreviations

AB	Autoinducer bioassay medium
AHL	acylated homoserine lactone
Amp	ampicillin
C12-HSL	N-3-oxododecanoyl homoserine lactone
C4-HSL	N-butyryl-L-homoserine lactone
DPD	4,5-dihydroxypentane-2,3-dione
esbp	extracellular solute-binding protein
GFP	green fluorescent protein
Gm	gentamicin
Km	kanamycin
LB	Luria-Bertani medium
MB	Marine broth
MCS	multiple cloning site
OD	optical densisty
PBS	phosphate saline buffer
R6K	R6K origin of replication
RP4mob	origin of transfer
RT	room temperature
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl methionine
SDM	*Shewanella* Defined Minimal medium
Sm	streptomycin
SM	*Shewanella* medium
SRH	S-ribosyl-homocysteine
Tc	tetracycline
tonBr	TonB dependent receptor
Tp	trimethroprim resistance
wt	wild-type

List of chemicals

L-arginine	Sigma
$CaCl_2$	Merck; M=147.02
Chromazurol S (CAS)	Sigma; M=605.29; 65 % dye content
$CoSO_4$-7-hydrat	Fluka; M = 281.1
$CuSO_4$-5-hydrat	Merck; M = 249.68
ethylene diamine tetraacetic acid (EDTA)	Merck, M = 372.24
ethidium bromide	
$FeCl_3$-6-hydrat	Fluka, M=270.3
$FeSO_4$	Merck, M = 278.02
Na-L-glutamate	Merck
Glycerol	Roth
H_3BO_3	Merck; M = 61.83
Hexadecyltrimethylammonium(HDTMA)	Sigma; M=364.46
K_2HPO_4	Merck; M=174.18
KCl	Roth; M = 74.55
KH_2PO_4	Merck; M=136.09
$MgCl_2$-6-hydrat	Riedel de Haën; M = 203.3
$MgSO_4$-7-hydrat	Roth; M = 246.48
$MnSO_4$	Fluka; M = 169.02
Na_2MoO_4-2-hydrat	Sigma; M = 241.95
Na_2SeO_4	Sigma; M = 188.9
NaCl	Roth; M = 58.44
$NaHCO_3$	Merck; M=84.01
$(NH_4)_2SO_4$	Roth; M=132.14
$Ni(NH_4)_2(SO_4)_2$	Fluka; M = 395
DL-serine	Merck
$ZnSO_4$-7-hydrat	Merck; M = 287.54

1 Introduction

A bacterium can successfully sustain its species, if it skilfully adapts to a wide range of niches that may already contain a complex consortium of other species. If the bacterium manages to fit in and contributes to the effectiveness of the community, it has a better chance of surviving. To respond appropriately to a community, several bacteria use signals released into the environment. The signalling process is named bacterial communication, also known as quorum sensing. In the last decades, quorum sensing has been discovered to regulate a number of important bacterial features (Hardie *et al.*, 2005)

1.1 Quorum sensing

Quorum sensing is a regulatory mechanism of gene expression, which induces bacteria to change their behaviour as the population reaches a particular cell-density, the so-called quorum. It functions through a cell-to-cell signalling molecule, also known as autoinducer, which is secreted into the surrounding environment. At low cell-densities the autoinducer is at low concentration. During growth, the concentration increases and thereby reflects the bacterial cell-density. At a critical threshold concentration, the autoinducer is recognized by the cells and the expression of specific genes of the cell become up- or downregulated (Fuqua *et al.*, 1994).

1.1.1 Alternative interpretations

Several contemporary researchers have thought that the conventional interpretation of bacterial communication is too simple and that this model does not encompass all forms of the process. Two of them attempted to redefine and to amend the quorum sensing model and represented two alternative interpretations: the diffusion sensing and the starvation sensing.

The idea of **diffusion sensing** originated from Redfield (Redfield, 2002). In this interpretation, the cells release autoinducers in order to assess the diffusion-rate in the immediate environment but not the cell-density. It is supported by the observation that autoinducers are low-cost products and they usually upregulate the secretion of substances

that have high energy cost for synthesis, e.g. exoenzymes. These costly substances could diffuse away and would become lost to the cell at high diffusion rates. If the cheap autoinducer accumulates in the immediate environment of the cells, it conveys to the cell low diffusion-rates and that the secretion of costly substances is favourable since these substances, just like autoinducers, will not diffuse away but remain in the immediate environment of the cell.

Another interpretation concerning the role of some specific autoinducers was **starvation sensing** proposed by Lazazzera (Lazazzera, 2000). In this interpretation, Quorum sensing pathways may converge with starvation-sensing pathways and some autoinducers are interconnected with the physiology of the cell. These autoinducers convey starvation and regulate the entry into the stationary phase. The arguments are based on the peculiarities of the competence-stimulating peptide (CSP) of *Bacillus subtilis*. The transcription of CSP is controlled by a sigma factor active under starvation, and as a result, more CSP is produced under nutrient limitation. In response, CSP triggers the utilization of alternative energy sources. CSP also upregulates competence, a typical quorum sensing phenotype, but it does it in concert with another autoinducer, and compared to the other autoinducer, its contribution is weaker. Another example was represented, for instance, by the autoinducer of *Pseudomonas aeruginosa*, an autoinducer which activates the sigma factor that regulates the entry into the stationary phase.

1.1.2 Regulated phenotypes

Quorum sensing regulates a wide range of phenotypes. Typical quorum sensing controlled phenotypes are those which are most efficiently expressed at high cell-density, or support the cell in adapting to and exploiting a particulate niche (Swift *et al.*, 2001). They involve the development of multicellular structures, production of extracellular substances, bioluminescence, motility and swarming, and DNA transfer. These phenotypes have been already summarized (Swift *et al.*, 2001), from which review a short exemplification will follow.

Using microarray profiling of the quorum sensing mutants, several other processes were regulated by quorum sensing. In particular cases, the ratio of the regulated genes was so high, that the quorum sensing system was stated to have global control over the cell (Waters & Bassler, 2005). In one of the best studied species, in *P. aeruginosa,* approximately 6 % of the genes is quorum sensing controlled (Schuster *et al.*, 2003).

The most common **multicellular structure** formed by bacteria is **biofilm**. Biofilms are ubiquitous and defined as a matrix-enclosed bacterial population adherent to each other and to surfaces. Bacteria form biofilm under nutrient limitation, a condition under which they transform themselves from planktonic into sessile life mode. Biofilm formation can be divided into five stages: reversible adhesion, irreversible adhesion, the appearance of aggregates, the formation of mature structures and the detachment of the cells (Figure 1-1). In the mature structures, the bacteria are embedded in an extracellular polymeric matrix (EPS). The matrix protects the bacteria against harmful agents like antibiotics, and contains water channels in which dissolved nutrients and autoinducers can circulate (Costerton *et al.*, 1995)

Biofilm formation is the most commonly investigated quorum sensing phenotype. quorum sensing can affect both the structure and biomass of the biofilm. As in many bacterial species, in *Pseudomonas aeruginosa*, a quorum sensing mutation disrupts the biofilm structure (Figure 1-1).

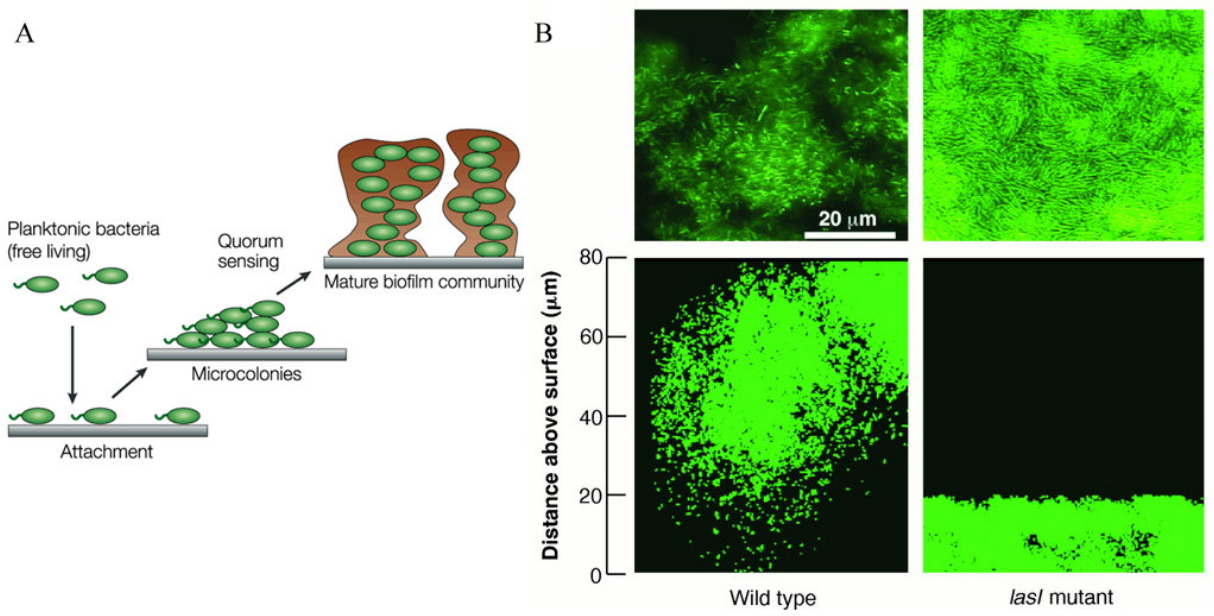


Figure 1-1. Biofilm development in Gram negative bacteria (A) and quorum sensing effect on *Pseudomonas aeruginosa* biofilm (B)
Biofilm development (Fuqua & Greenberg, 2002): after irreversible adhesion of bacteria, microcolonies are formed. The microcolonies evolve into mushroom-like protrusions by continuous supply of fresh medium.
The effect of Quorum sensing mutation on the mature structure of *P. aeruginosa* biofilm (Davies *et al.*, 1998): The cells were labelled with green fluorescence protein by harbouring an expression vector. Epifluorescence micrographs at the top and confocal laser scanning images at the bottom line illustrate the top view and the profile of biofilms respectively. The biofilm of the wildtype and quorum sensing mutant (*lasI* mutant) are shown in the left and right pictures respectively. The wildtype cells stratified into clusters, whereas the *lasI* mutant covered the surface with an even layer.

Another multicellular structure regulated by quorum sensing is the formation of **fruiting bodies** by *Myxobacteria*. Fruiting bodies are formed by bacteria that starve, and thereby, move into a common direction, aggregate into a well-defined form, differentiate into resistant spores and finally disperse into the air (Sozinova *et al.*, 2006).

Production of extracellular substances. Extracellular substances regulated by quorum sensing can be **enzymes, toxins, antibiotics, siderophores and some pigments**, which can aid the pathogenesis of the organism and the establishment in a niche. The exoenzymes digest large, often insoluble extracellular polymers, e.g. cellulose, to facilitate the uptake of breakdown products. Siderophores are iron chelators supporting the competition for iron, and they can also function as toxins, antibiotics and potentially autoinducers. Toxins, antibiotics and pigments inhibit competitors or weaken the host immune system. In *Pseudomonas aeruginosa*, quorum sensing regulates a wide-range of extracellular substances. These substances include exoenzymes, as elastase and alkaline protease, toxin, as exotoxin A, the phenazine pigment, pyocyanin, and the siderophores, pyoverdin and pyochelin. In *Erwinia carotovora*, quorum sensing controls the production of the carbapenem antibiotic, and in *Serratia marcescens*, it controls the production of prodigiosin, a pigment with antimicrobial and immunosuppressive activity.

Bioluminescence. Bioluminescence in bacteria is generated by the luciferase enzyme encoded on the *lux* genes using energy and oxygen. In *V. fischeri*, bioluminescence supports symbiosis with the Hawaiian bobtail squid. The bacterial cells colonize in the light organ of the squid, and produce light aiding the camouflage of the squid. In exchange, the squid supplies nutrients for the bacteria. The signalling of *Vibrio fischeri* was the first elucidated, quorum sensing system (Eberhard *et al.*, 1981; Nealson *et al.*, 1970) and it illustrates, how quorum sensing controls the switch in a species from the free-living into the symbiotic life mode.

Motility and swarming. Motility using flagella and swarming using the twitching motility via type IV pili are required for dispersal of a bacterium. In *Yersinia tubercolosis*, quorum sensing upregulates the main flagellar components in a temperature-dependent manner, and in *Serratia liquefaciens*, it upregulates swarming.

DNA transfer. DNA transfer regulated by quorum sensing compromised of conjugal transfer or elevated competence. Conjugal transfer is DNA transfer from a donor to a recipient cell through physical contact (pili). In two root-colonizing bacteria, *Agrobacteria* and *Rhizobia*, quorum sensing regulates conjugal transfer of a large plasmid. This plasmid encodes the quorum sensing components, proteins for conjugation and specific enzymes necessary for the specific niche, the root. Competence is the ability of a bacterium to take

up free DNA from the environment. According to our present knowledge, quorum sensing upregulates competence in all Gram-positive bacteria (Kleerebezem *et al.*, 1997).

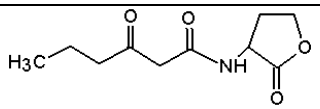
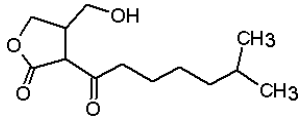
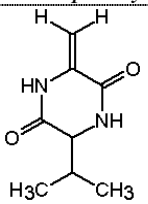
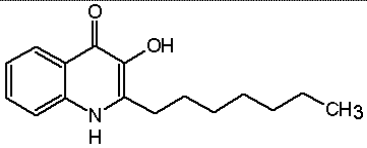
1.1.3 Autoinducer structures

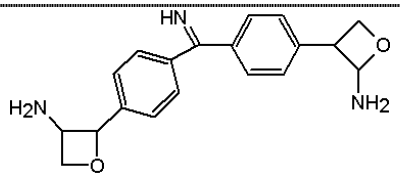
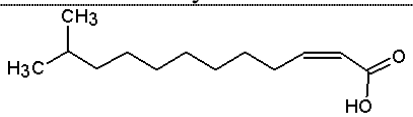
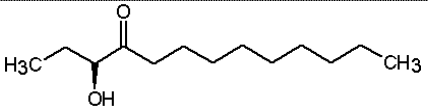
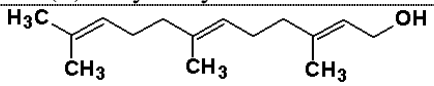
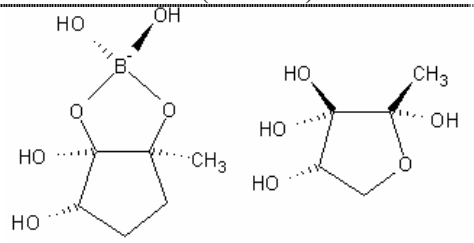
A wide range of chemical compounds function as autoinducers. The two best-studied autoinducer group of compounds are the acylated homoserine lactones (AHL) of Gram negative bacteria and post-translationally modified peptides in Gram positive bacteria. Furthermore autoinducers, with various other structures have been identified (Table 1-1).

The autoinducers can be sensed by two ways. They extracellularly stimulate a histidine sensor kinase or they are uptaken and interact with transcriptional regulators.

All of these autoinducers are in majority species-specific or sensed by only closely related species, except AI-2.

Table 1-1. Representative autoinducers in bacteria.

Autoinducer	Structure	Species	Ref.
AHLs	 N-(3-oxo-hexanoyl)L-HSL of *Vibrio fischeri*	Gram negative species	(Gonzalez & Keshavan, 2006)
Oligopeptides	S-G-G-L-S-T-F-F-R-S-F-T-Q-A-L-G CSP of *Streptococcus mutans*	Gram positive species	(Waters & Bassler, 2005)
γ-butyrolactones	 A factor of a *Streptomyces griseus*	*Streptomyces* species	(Takano, 2006;Waters & Bassler, 2005)
Diketopiperazines	 cyclo (ΔAla-L-Val) of *Pseudomonas aeruginosa*	*Pseudomonas* species	(Gonzalez & Keshavan, 2006;Holden *et al.*, 1999)
Hydroxyquinolone	 2-heptyl-3-hydroxy-4-quinolone (PQS)	*Pseudomonas aeruginosa*	(Pesci *et al.*, 1999)

Siderophore	 Bradyoxetin	*Bradyrhizobium japonicum*	(Gonzalez & Keshavan, 2006;Loh *et al.*, 2002)
Diffusible signal factor (DSF)	 *cis*-11-methyl-2-dodecenoic acid of *Xanthomonas. campestris*	*Xanthomonas, Xylella*	(Gonzalez & Keshavan, 2006;Wang *et al.*, 2004)
Cholerae auto-inducer (CAI-1)	 (S)-3-hydroxytridecan-4-one	*Vibrio cholerae*	(Higgins *et al.*, 2007)
Fornesol (fatty acid)	 3,7,11-trimethyl-2,6,10-dodecatriene-1-ol (fornesol)	*Candida albicans*	(Shchepin *et al.*, 2003)
Autoinducer-2	 AI-2 compounds	Many species	(Chen *et al.*, 2002;Miller *et al.*, 2004)

1.2 Autoinducer-2

1.2.1 History

Autoinducer-2 (AI-2) was discovered in the early nineties by Bassler as a quorum sensing signal of the marine bacterium, *Vibrio harveyi*, in which AI-2 was found to trigger luminescence in concert with autoinducer-1 (AI-1) (Bassler *et al.*, 1994). In the course of this research, *V. harveyi* quorum sensing mutants were constructed, mutants which are named BB170 and BB886 and detect only AI-2 and only AI-1 respectively. In a previous study, the luminescence of *V. harveyi* was shown to be induced by many, also non-*Vibrio* species (Greenberg *et al.*, 1979). Based on the previous observation, BB170 and BB886 were tested for this cross-species induction and interestingly particularly BB170, the AI-2 sensor was induced, but only rarely BB886, the AI-1 sensor. It was concluded that AI-2 is an interspecies communication signal, while AI-1 is a species-specific signal of *V. harveyi* (Bassler *et al.*, 1997)

In further studies, the gene responsible for AI-2 production in *V. harveyi* was identified and it was named *luxS* (*lux*-luminescence, S-syntheses) (Surette *et al.*, 1999). In addition, *luxS* homologs were identified in several Gram negative and Gram positive bacteria and in all of them, AI-2 activity was measured with the *V. harveyi* BB170, AI-2 sensor strain. In each case, knockout mutation of the *luxS* gene eliminated the AI-2-activity and reintroduction of *luxS* restored it. Even a heterologously expressed *luxS* gene restored the AI-2-activity and thus the *luxS* homologs were exchangeable.

In the light of all these observations, it was suggested AI-2 could be a universal signal molecule of bacteria in their natural environments and AI-2 might represent the "Bacterial Esperanto" (Bassler, 1999). This idea aroused the interest of the researchers in quorum sensing and they started to investigate the role of AI-2 in the quorum sensing system of different bacteria.

1.2.2 Synthesis

1.2.2.1 Synthesis via the Pfs/LuxS enzymatic pathway

AI-2 is synthesized by the LuxS enzyme, which participates in the activated methyl cycle. The activated methyl cycle is present in each free-living organism and has a central role in the metabolism. In the cycle, all the enzymes are well-conserved, and thus they must serve the same function in each bacterium (Winzer *et al.*, 2002a).

The starting compound in the activated methyl cycle is S-adenosyl-methionine (SAM), which is the general methyl donor in the cell and donates its methyl group to diverse cellular components including DNA, RNA, proteins and certain metabolites. Upon methyl release, SAM is converted to S-adenosyl-homocysteine (SAH), which is a toxic compound and needs to be recycled.

For recycling SAH, organisms can possess two possible pathways (Figure 1-2) consisting of one step and two steps respectively. Only the two-step pathway results in AI-2, and this way is exclusive to *Bacteria*, while the alternative one-step pathway is used in all domains, in some *Bacteria*, in all *Eukarya* and in all *Archaea*.

The two-step pathway contains the Pfs and the LuxS enzymes: the Pfs enzyme converts SAH into S-ribosyl-homocysteine (SRH) and adenine; the LuxS enzyme cleaves SRH into homocysteine and 4,5-dihydroxy-2,3-pentadione (DPD). Homocysteine, the product of the LuxS enzyme, remains in the cycle and is converted by MetE or MetH (5-methyltetrahydro-pteroyl-triglutamate-homocysteine methyltransferase or 5-methyl-tetrahydrofolate-homocysteine methyltransferase) to methionine. Methionine is then

converted back by MetK (SAM synthetase) into SAM thereby returning to the starting compound. DPD, the other product of the LuxS enzyme, forms AI-2, and should diffuse into the environment.

The one-step pathway contains S-adenosyl-homocysteine hydrolase (SahH) that converts SAH into homocysteine and adenosine, but without production of AI-2.

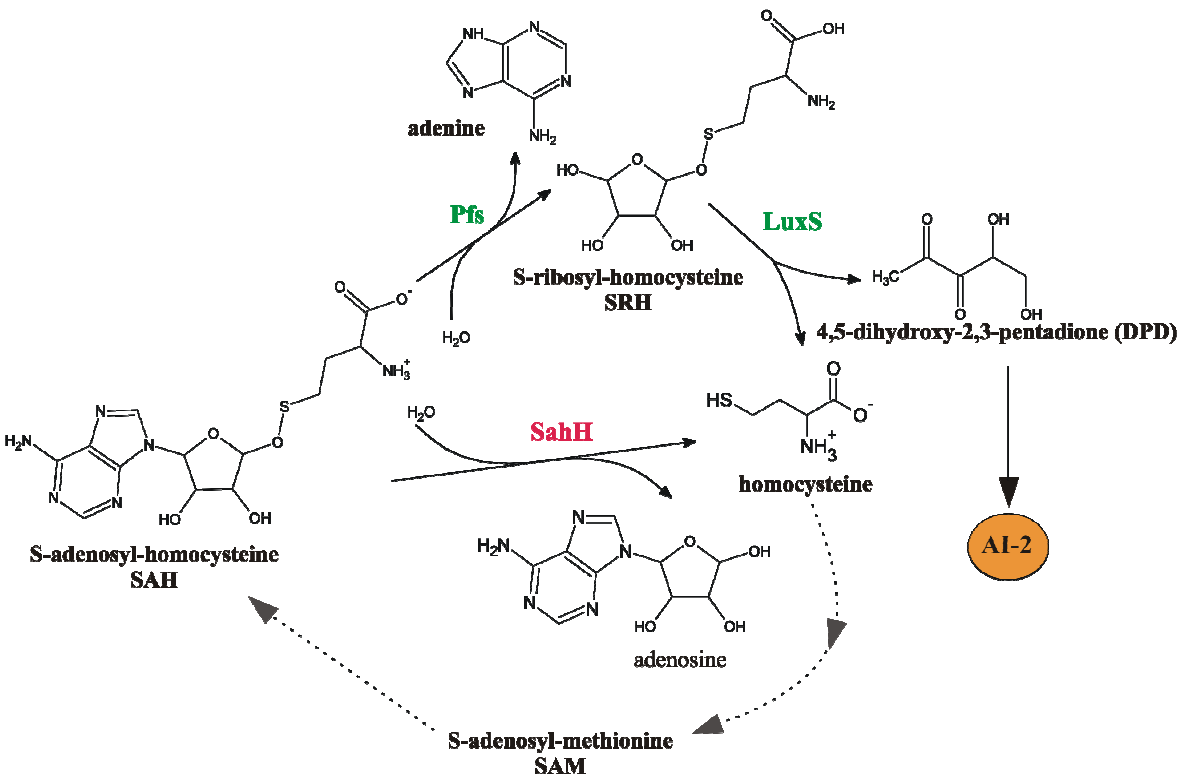


Figure 1-2. AI-2 producing and alternative enzymatic pathway for SAH recycling in the activated methyl cycle.
AI-2 is produced by two enzymatic steps (green enzymes) during the recycling of S-adenosyl-homocysteine (SAH), a toxic compound, which is derived from S-adenosyl-methionine (SAM). The first enzyme, Pfs converts SAH into adenine and S-ribosyl-homocysteine (SRH). Then, the second enzyme, LuxS, cleaves SRH into dihydroxy pentadione (DPD), the pre-form of AI-2 and homocysteine. Homocysteine is then reconverted into SAM, the starting compound of the cycle.
The alternative pathway for SAH recycling consists of one enzyme, S-adenosyl-homocysteine hydrolase (SahH), which cleaves SAH into homocysteine and adenosine without AI-2 production. The illustration is adapted and modified from Sun and coworker (Sun *et al.*, 2004)

1.2.2.2 AI-2 forms: epimeric DPD derivatives

AI-2 is referred to two DPD derivatives, which are spontaneously formed and exist in chemical equilibrium in aqueous solutions (Miller *et al.*, 2004).

In an aqueous environment, DPD becomes spontaneously cyclisized and hydrated (Figure 1-3). The cyclic and hydrated DPD has two stereoisometric forms: S- and R-THMF ((2R,4S)- and (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran). The stereoisomers are in a chemical equilibrium and can permanently interconvert through the open form. Due to the spatial arrangement of the hydroxyl groups, S-THMF binds borate, a ubiquitous trace metal, and forms S-THMF-borate. In contrast, R-THMF remains without borate.

AI-2 compounds are heat resistant to 80 °C, stable at acidic pH but labile at basic pH (Surette & Bassler, 1998).

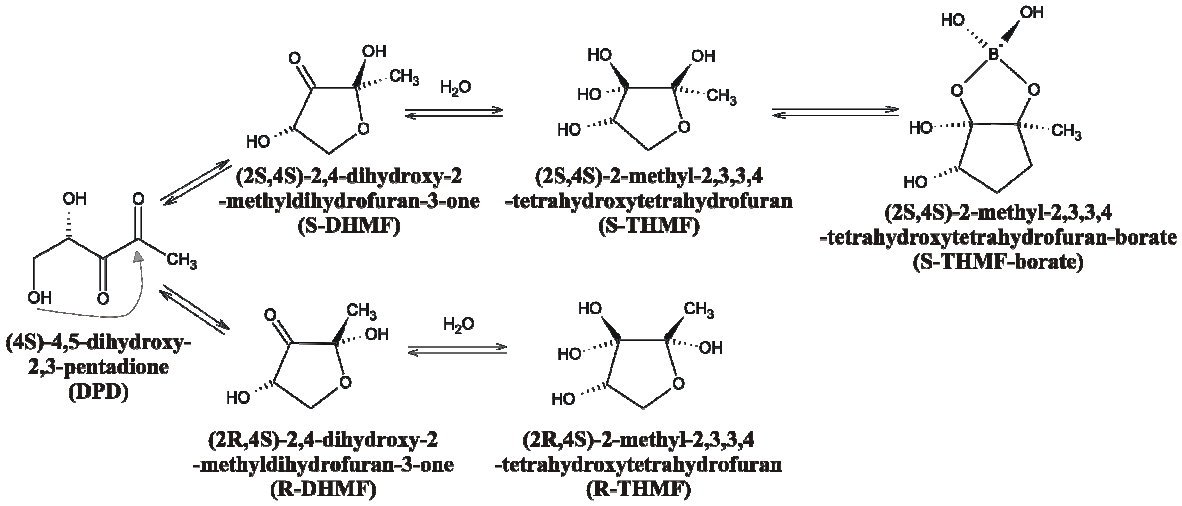


Figure 1-3. Two forms of AI-2 derived from DPD.
DPD spontaneously cyclisizes into two epimeric furanones. In an aqueous environment, the furanones become hydrated. The one stereoisomer, S-THMF, binds borate spontaneously due to its spatial arrangement, and S-THMF-borate is formed. The other, R-THMF, remains unborated. The illustration is adapted from Miller and co-worker (Miller *et al.*, 2004)

The *Vibrio* species sense S-THMF-borate by the so-named LuxP protein (Chen *et al.*, 2002), whereas *Salmonella typhimurium* and *Escherichia coli*, recognize R-THMF by binding to LsrB protein (Miller *et al.*, 2004). However, each species can react to AI-2 produced by other species, because the two forms are in equilibrium and interconvert.

1.2.3 Signal response

To date, two types of response mechanisms to AI-2 have been identified: a signalling cascade in *V. harveyi* and *V. cholerae*, and an uptake-phosphorylation process of *Salmonella typhimurium* and *Escherichia coli*.

1.2.3.1 *Vibrio harveyi*

Regulatory role.

In *V. harveyi*, AI-2 in combination with two other autoinducers upregulate bioluminescence (Bassler *et al.*, 1993) and a number of other phenotypes, e.g. metalloprotease production, and downregulate type III secretion (Mok *et al.*, 2003), siderophore production and rugose colony morphology (Lilley & Bassler, 2000)

Signalling cascade of *Vibrio harveyi*.

AI-2 is one of the three autoinducers in *Vibrio harveyi* (Figure 1-4) (Tu & Bassler, 2007). The other signals are AI-1 (N-(3-hydroxybutanoyl)-L-homoserine lactone), which is a species-specific autoinducer and synthesized by LuxM (Bassler *et al.*, 1993), and the recently discovered CAI-1-like autoinducer, which is a probable genus-specific autoinducer and synthesized by the CqsA homolog (cholerae quorum sensing autoinducer, since it was discovered in *V. cholerae*) (Henke & Bassler, 2004b). Both the universal AI-2 and the species-specific AI-1 are capable of stimulating bioluminescence, but AI-2 has much less influence than AI-1 (Freeman *et al.*, 2000)

The signals are sensed by histidine sensor kinases residing in the cell membrane. A histidine sensor kinase can act as a phosphatase or as a kinase and if either of the modes is activated, this information is conveyed by a phosphorylation cascade to transcriptional regulators that induce or repress gene expression. AI-2 is first bound by the LuxP, a receptor protein in the periplasmic protein, and then sensed by LuxQ histidine sensor kinase. AI-1 and CAI-1 are sensed by the LuxN and the CqsS histidine sensor kinases respectively (Freeman *et al.*, 2000; Henke & Bassler, 2004b).

At low cell-density, when autoinducers are at low concentration, LuxQ, LuxN and CqsS act as kinases and transfer a phosphate group to the LuxU phosphorelay protein, which passes the phosphate group to the LuxO regulator. Phospho-LuxO together with the RpoN sigma factor activate the expression of non-coding small regulatory RNAs (sRNAs), also named Qrr (quorum regulatory RNAs). An RNA chaperone, Hfq, promotes base-pairing of the sRNAs and the mRNA of *luxR*, the master regulator of *V. harveyi*. The sRNA-mRNA

complex is degraded, and therefore the mRNA of *luxR* cannot be translated. In the absence of LuxR, luminescence is not induced. In contrast, other genes are expressed, like the genes of typeIII secretion, siderophore production and the rugose colony phenotype.

At high cell-density, when autoinducers are at high concentration, LuxQ, LuxN and CqsS act as phosphatases and transfer the phosphate group into the opposite direction, and therefore, LuxU and LuxO are dephosphorylated. The dephosphorylated LuxO is inactive and does not induce the expression of sRNAs. In the absence of sRNAs, the LuxR, the master regulator, is expressed and initiates the expression of genes responsible for bioluminescence and the production of metalloprotease (Tu & Bassler, 2007).

The use of sRNAs facilitates gene regulation gradually according to the current autoinducer level (Tu & Bassler, 2007), and thus this quorum sensing system is not activated at the threshold concentration.

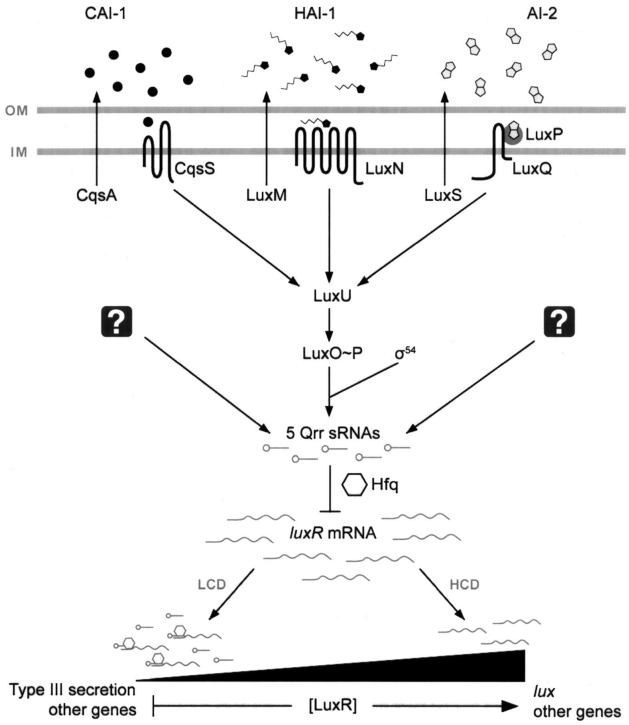


Figure 1-4. Model of the *V. harveyi* Quorum sensing system (Tu & Bassler, 2007).
Three parallel sensory systems converge to regulate quorum sensing gene expression by controlling levels of the master transcriptional regulator, LuxR. The three autoinducers are AI-2 (double pentagons), HAI-1 (pentagons with side chains) and CAI-1 (circles), which are sensed by the LuxQ, LuxN and CqsS, histidine sensor kinases respectively. At low autoinducer concentration, the histidine sensor kinases act as kinases and allow the transfer of a phosphate group to LuxU, phophorelay protein, which transfers it to LuxO. LuxO-P together with RpoN (σ^{54}) induces the expression of the Qrr sRNAs (lollipops), which indirectly regulate LuxR protein levels by destabilizing *luxR* mRNA (wavy lines), a process that is mediated by the RNA chaperone Hfq (hexagons). At high autoinducer concentration, the sensor kinases act as phosphatases and let the phosphate group transfer into the opposite direction, thereby dephosphorylating LuxO, which then does not activate the Qrr sRNas. The multiple Qrr sRNAs produce an increasing gradient of LuxR protein as the cells transition from low to high autoinducer concentration, i.e. cell-density. Question marks denote putative regulators that control *qrr* expression. (OM) Outer membrane; (IM) inner membrane; (LCD) low cell density; (HCD) high cell density.

1.2.3.2 *Salmonella typhimurium* and *Escherichia coli*

Regulatory role.

In *S. typhimurium* and in *E. coli*, the role of AI-2 is less defined.

Although it is known, that the knockout mutation of the AI-2 synthesis gene, i.e. *luxS* gene, caused impaired biofilm formation and pleiotropic effects related to stress in *S. typhimurium* and in *E. coli*, respectively, AI-2 has not restored these phenotypes to the wildtype state (Ahmer, 2004; De Keersmaecker *et al.*, 2005).

To date it is proven, that AI-2 regulates its own uptake and phosphorylation. This fact was interpreted by authors differentially. Some authors stated that these bacteria consume AI-2, since uptake and phosphorylation are tightly linked with the physiological state of the cell (Winzer *et al.*, 2002b), while other authors suggested that these bacteria may remove AI-2 to quench the quorum sensing system of competitive, gastroenterical pathogens, like *V. cholerae* (Xavier *et al.*, 2007). An alternative theory has recently been published, in which it was proposed that enterics use AI-2 as an autoinducer before uptake and phosphorylation (Li *et al.*, 2007).

Since, *E. coli* was more intensively investigated than *S. typhimurium*, the following description reviews the results about *E. coli*.

Signal response of *Escherichia coli.*

AI-2 regulates genes responsible for its uptake and phosphorylation (Figure 1-5). These functions are encoded on the *lsrACDBFG* (*luxS* regulated) operon and by the *lsrR* and *lsrK* genes, which are located immediately upstream of the *lsr* operon but transcribed into the opposite direction (Taga *et al.*, 2001; Taga *et al.*, 2003). The *lsr*R and *lsr*K genes encode a transcriptional repressor and a kinase, respectively.

In the exponential phase, AI-2 accumulates extracellularly. The *lsrACDBFG* operon and *lsrK* gene responsible for uptake and phosphorylation of AI-2 are not expressed, since they are inhibited by the binding of LsrR and catabolit repression (Wang *et al.*, 2005; Xavier & Bassler, 2005). Towards the end of the exponential phase, when extracellular AI-2 concentration is high, AI-2 appears to enter the cell by an unknown mechanism, to remain unphosphorylated, and to bind to the LsrR repressor, which could then release the repression of typical quorum sensing regulated genes (Li *et al.*, 2007).

At the end of the logarithmic phase, LsrK kinase is expressed and phosphorylates AI-2 in the DPD form (Xavier *et al.*, 2007). Phospho-DPD binds to LsrR repressor, which then

releases the repression of the *lsrACDBFG* operon encoding an ABC transporter and the LsrF and the LsrG enzymes.

The ABC-transporter consists of LsrACDB proteins: the LsrB periplasmic protein binds AI-2, and the other components, LsrACD, transport it into the cell. As AI-2 enters the cell, LsrK phosphorylates it in the DPD form. The phospho-DPD binds to LsrR, which is then release the *lsr* operon, and thus phospho-DPD indirectly induces the uptake and phosphorylation of all external AI-2.

Surplus of phospho-DPD is probably converted by LsrF and LsrG enzymes to other molecules (Wang *et al.*, 2005; Xavier & Bassler, 2005). LsrG appears to act first and catalyses the cleavage of phospho-DPD into 2-phosphoglycolic acid and another three-carbon compound (Xavier *et al.*, 2007).The exact function of LsrF is unknown, but it shares homology with putative aldolases.

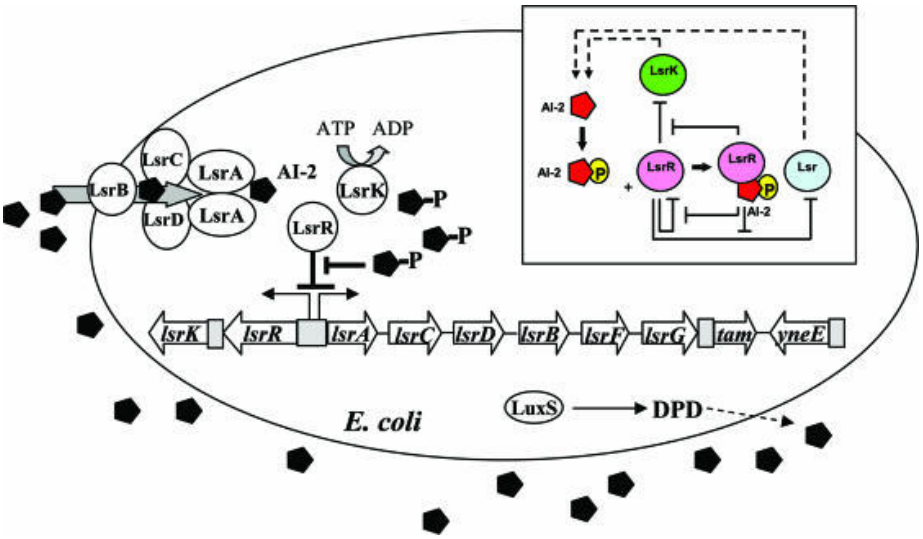


Figure 1-5. Model for uptake and phosphorylation of AI-2 in *E. coli* (Li *et al.*, 2007).
In the exponential phase, LsrR repressor inhibits the expression of the *lsrACDBFG* operon and the *lsrK* gene. At the end of the logarithmic phase, LsrK kinase is expressed and phosphorylates AI-2. Phospho-AI-2 binds to the LsrR repressor, which then releases the expression of the *lsrACDBFG* operon. LsrACDB encodes for an ABC transporter, from which LsrB binds extracellular AI-2, while LsrACD components transport AI-2 into the cell. All internal AI-2 is phosphorylated then by LsrK, and phospho-AI-2 permanently derepresses the *lsr* operon by binding to LsrR thereby resulting in the internalization of all external AI-2. The proposed function of LsrF and LsrG is to decompose phospho-DPD.

1.2.3.3 Other species: phenotypes of *luxS* mutants and their complementation

Several other species were investigated which have the *luxS* gene and potentially communicate via AI-2. In these species, *luxS* mutants were constructed and characterized by phenotypic changes.

The *luxS* mutants exhibited several quorum sensing-related phenotypes. They had most often biofilm deficiency, but many of them were defective in toxin production or were impaired in their infectivity in *in vivo* systems. Some of them had differential growth under particular conditions, showed different expression of genes responsible for iron acquisition, had problems with motility or reacted differentially to stress factors, like antibiotics, heat, pH or hydrogen peroxide. A few of them also showed alteration in intracellular processes, like metabolism or expression of transcriptional regulator, or they even showed global alterations in gene expression studies.

The *luxS* mutant phenotype is not necessarily caused by the absence of quorum sensing via AI-2. It can be caused either by the absence of AI-2 or by the disruption of the activated methyl cycle. Therefore, to state that AI-2 is involved in one phenotype as a potential quorum sensing signal, it is crucial to restore the *luxS* mutant to the wildtype phenotype by complementing with AI-2. Still, numerous *luxS* mutants were characterized but not complemented. Other *luxS* mutants were complemented on five different way: with *luxS* gene expressed in *trans*, with culture supernatant of the wildtype strain, with partially purified AI-2 (Sperandio *et al.*, 2003), with enzymatically synthesized AI-2 (Schauder *et al.*, 2001a) and with chemically synthesized DPD (De Keersmaecker *et al.*, 2005; Semmelhack *et al.*, 2005).

The complementation with the *luxS* gene expressed in *trans* and that with the culture supernatant of the wildtype strain are not specific enough to AI-2. The complementation with in *trans* expressed *luxS* gene also restores the metabolic defect not just the absence of AI-2. The complementation with a culture supernatant of the wildtype strain can contain besides AI-2 other factors that restore the wildtype phenotype. In Table 1-2, only the *luxS* mutants are summarized that were complemented with either partially purified or enzymatically or chemically synthesized AI-2. As shown, AI-2 restoration was in a few cases successful.

In summary, there are several indications, that AI-2 may be a signalling molecule, since mutants of AI-2 synthesis had quorum sensing-related phenotypes, which phenotypes were partially restored by complementing AI-2. However, AI-2 seems to convey different information to each species, and in number of species, the role of AI-2 is largely unexplored or it has not been investigated.

Table 1-2. Phenotypes of luxS mutants in diverse organisms and their complementation.

Strain	Phenotype	Complementation	Reference
Aggregatibacter actinomycetemcomitans	– Reduced biofilm growth in flow-chamber	luxS gene in trans and partially purified AI-2 : biofilm restored	Shao *et al.*, 2007
Helicobacter pylori	– Reduced motility	DPD (0.1 mM) modulated flagellar gene transcription	Rader *et al.*, 2007
Porphyromonas gingivalis	– Differential expression of genes responsible for iron acquisition (tlr, kgp, hmuR, fetB, feoB1, ferritin)	partially purified AI-2: restored hmuR and tlr	James *et al.*, 2006
Staphylococcus epidermidis	– Differential expression: metabolism genes (sugar, nucleotide, amino acid, and nitrogen) and virulence-associated genes (lipase and bacterial apoptosis protein) – Reduced level of pro-inflammatory phenolsoluble modulin toxins	Enzymatically synthesized AI-2: restored	Li *et al.*, 2008
Streptococcus anginosus	– Higher susceptibility to erythromycin and ampicillin	DPD (1.5-1.8 nM) restored	Ahmed *et al.*, 2007
Streptococcus intermedius	– Downregulation of virulence-associated genes (hialuronidase, intermedilysin) – Reduced (5-fold) haemolytic activity	DPD (0.4-32 nM): haemolytic activity partially restored	Pecharki *et al.*, 2008
Streptococcus mutans	– Differential expression of diverse genes (30 % of the genome)	DPD: 59 genes (10 % of regulated genes) restored	Sztajer *et al.*, 2008
Streptococcus oralis	– Reduced biofilm in co-culture with *Actinomyces naeslundii*	DPD (0.8-0.08): the mixed biofilm was restored but not the single-species biofilm	Rickard *et al.*, 2006
Lactobacillus reuteri	– Thicker biofilm on plastic surfaces in flow-chamber	Purified AI-2: not restored the biofilm on plastic surfaces	Tannock *et al.*, 2005
Lactobacillus rhamnosus	– Reduced biofilm on polystyrene peg	DPD (0.5 nm-0.5 mM) : not restored	Lebeer *et al.*, 2007
Salmonella enterica	– Reduced biofilm formation on polystyrene pegs	DPD (78 µM): not restored	De Keersmaecker *et al.*, 2005

1.2.4 Pseudomonas aeruginosa

Pseudomonas aeruginosa belongs to the *Gammaproteobacteria*. It is present ubiquitously in nature and it is a broad-host-range pathogen and capable of infecting mammals, insects, nematodes and plants. In humans, it can cause blood, skin, eye and genitourinary tract infections. Nevertheless, it is particularly studied, because of causing chronic lung infections in cystic fibrosis patients (Swift *et al.*, 2001). Cystic fibrosis is a hereditary illness in Caucasians causing a defect in the chloride ion channel, which results in a more viscous mucous layer in the lung than normal. Bacteria attach to the viscous layer and cause periodical, serious lung inflammations dominated by *P. aeruginosa*. Sooner or later, the bacterial infection develops into chronic colonization by *P. aeruginosa* that is mortal in 80 % of the cases. Most cystic fibrosis patients die by 18 years of age (Cystic Fibrosis Foundation, www.cff.org) (Rowe *et al.*, 2005)

P. aeruginosa is capable of developing biofilms and producing a variety of exoproducts, which are controlled by its quorum sensing system and contribute to its virulence. The core of its quorum sensing is constituted of two LuxI/R type systems, named LasI/R and RhlI/R (Pesci & Iglewski, 1997) (Figure 1-6). The LasI, quorum sensing synthase, produces N-3-oxododecanoyl homoserine lactone (C12-HSL). At the critical concentration, C12-HSL binds to the LasR, transcriptional regulator. The activated LasR triggers the expression of virulence genes, the *rhlI/R* quorum sensing genes and the *lasI* autoinducer synthase gene. The triggered LasI enzyme produces more C12-HSL autoinducer thus creating a positive regulatory feedback loop. The induced RhlI synthesizes N-butyryl-L-homoserine lactone autoinducer (C4-HSL). At high autoinducer concentration, C4-HSL binds to RhlR, transcriptional regulator. The activated RhlR induce partially the same subset of virulence genes but also other genes and the *rhlI* autoinducer synthesis gene. The triggered RhlI synthesizes N-butyryl-HSL autoinducer (C4-HSL). At high autoinducer concentration, C4-HSL binds to RhlR, transcriptional regulator. The activated RhlR induces partially the same subset of virulence genes but also other genes and the *rhlI* autoinducer synthesis gene. The triggered RhlI produces more autoinducer thereby creating a self-generating loop in the Rhl quorum sensing system too. In recent years, many studies have focused on the quorum sensing system of *P. aeruginosa* and revealed a complicated regulatory network and global gene control (Schuster & Greenberg, 2007).

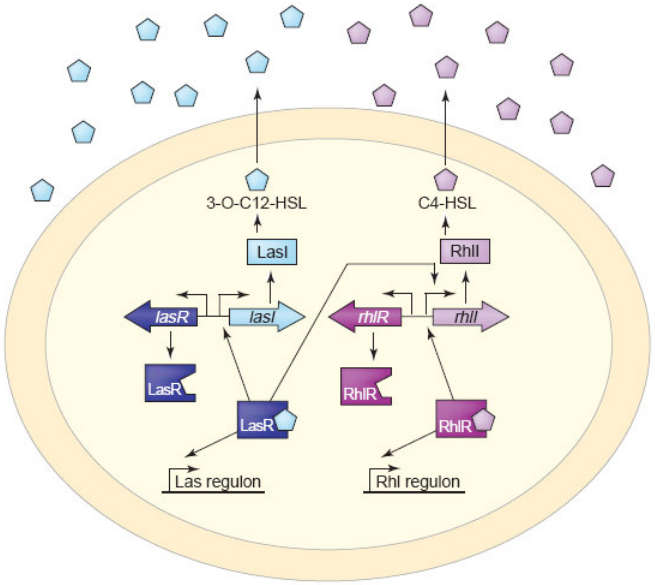


Figure 1-6. The quorum sensing regulatory circuit of *P. aeruginosa* (Henke & Bassler, 2004a).
The LasI enzymes synthesize the 3-O-C12-HSL, autoinducer. At critical threshold concentration, 3-O-C12-HSL binds to LasR regulator, which, in turn, activates the genes of the Las regulon and the *rhlI*/*rhlR* genes. The activated LasR induces also the *lasI* gene, and the expressed LasI produces more 3-O-C12-HSL, thereby creating a self-generating loop. The expressed RhlI synthesizes C4-HSL. At high autoinducer concentration, C4-HSL binds to RhlR, which, in turn, activates the genes of the Rhl regulon. The activated RhlR also induces the *rhlI* gene, and the expressed RhlI produces more C4-HSL, thereby creating a self-generating loop in the *rhl* system too.

However, *P. aeruginosa* does not have the *luxS* gene and therefore it cannot produce AI-2. Still, it is remarkable that *P. aeruginosa* contacts numerous species resident in the mouth and co-exists with species colonizing in the lung, and the majority of these species have the *luxS* gene and produce AI-2. The question was, if *P. aeruginosa* could eavesdrop the communication via AI-2 and enhance its virulence in response to AI-2.

Referring to this question, *P. aeruginosa* and oropharyngeal *Streptococcus* species were isolated from cystic fibrosis patients (Duan *et al.*, 2003). Firstly, the *Streptococcus* species were examined if they could communicate via AI-2, i.e. if they have the *luxS* gene and produce AI-2. The majority of the *Streptococcus* species had the *luxS* gene and produced AI-2 in a laboratory environment as well as in in-vivo environment, in cleared sputa and in bronchoalveolar lavage fluids of infected rats.

Then, *P. aeruginosa* was examined if it induces its virulence genes in response to the culture supernatant of the oropharyngeal *Streptococcus* species, and in case of confirmation, if *P. aeruginosa* induces its virulence genes in response to AI-2. For this purpose, they constructed luminescence reporter plasmids with promoters of 21 well-characterized virulence genes of *P. aeruginosa* and they introduced the plasmids into the clinical isolate of *P. aeruginosa*.

Nine virulence genes were induced by the culture supernatant of *Streptococcus* species and six out of the nine were slightly induced by AI-2. It was postulated that the host-resident *Streptococcus* species modulates the gene expression of *P. aeruginosa* partially via AI-2 signalling (Duan *et al.*, 2003). In case of confirmation, AI-2 would represent a potential autoinducer in *P. aeruginosa* in a clinically important habitat.

1.2.5 Phylogenetic distribution of the Pfs/LuxS and SAH pathway

The phylogenetic distribution of the Pfs/LuxS and SahH pathway has been investigated in fully sequenced organisms by Sun and co-worker (Sun *et al.*, 2004). At this time, 138 bacteria were fully-sequenced and they were mostly pathogenic, symbiotic or parasitic species (Figure 1-7).

Several species were analysed, nevertheless only four taxa could be included with numerous representatives. In the Gram negatives, the *Alphaproteobacteria* consistently used the SahH enzyme. The *Gammaproteobacteria* were mixed: *Enterobacteriales*, *Vibrionales*, *Pasteurellales* species and *Shewanella oneidensis* used the Pfs/LuxS way, while *Pseudomonadales* and *Xanthomonadales* species used the SahH way. In the Gram positive bacteria, the *Actinobacteria* used the SahH, while the *Firmicutes* used the Pfs/LuxS ways consistently. Only two species contained both of the pathways: *Escherichia blattae*, *Bifidobacterium longum*, and only symbionts, intracellular parasites contained neither of the pathways: Mollicutes or Chlamydiates.

Therefore, the studied bacterial species used either of the pathways, very rarely both or none of them. Members of the same phylogenetic group usually used the same pathway. They concluded the presence of the Pfs/LuxS or SahH pathway could be determined by the phylogenetic position of an organism.

However, 138 bacteria is a small segment for the bacterial world and these bacteria were biased with respect to their life mode. The question was: Do *non*-pathogenic species also have the potential to communicate via AI-2 and the same phylogenetic distribution of Pfs/LuxS pathway as the pathogenic bacteria, or do they have it less frequently?

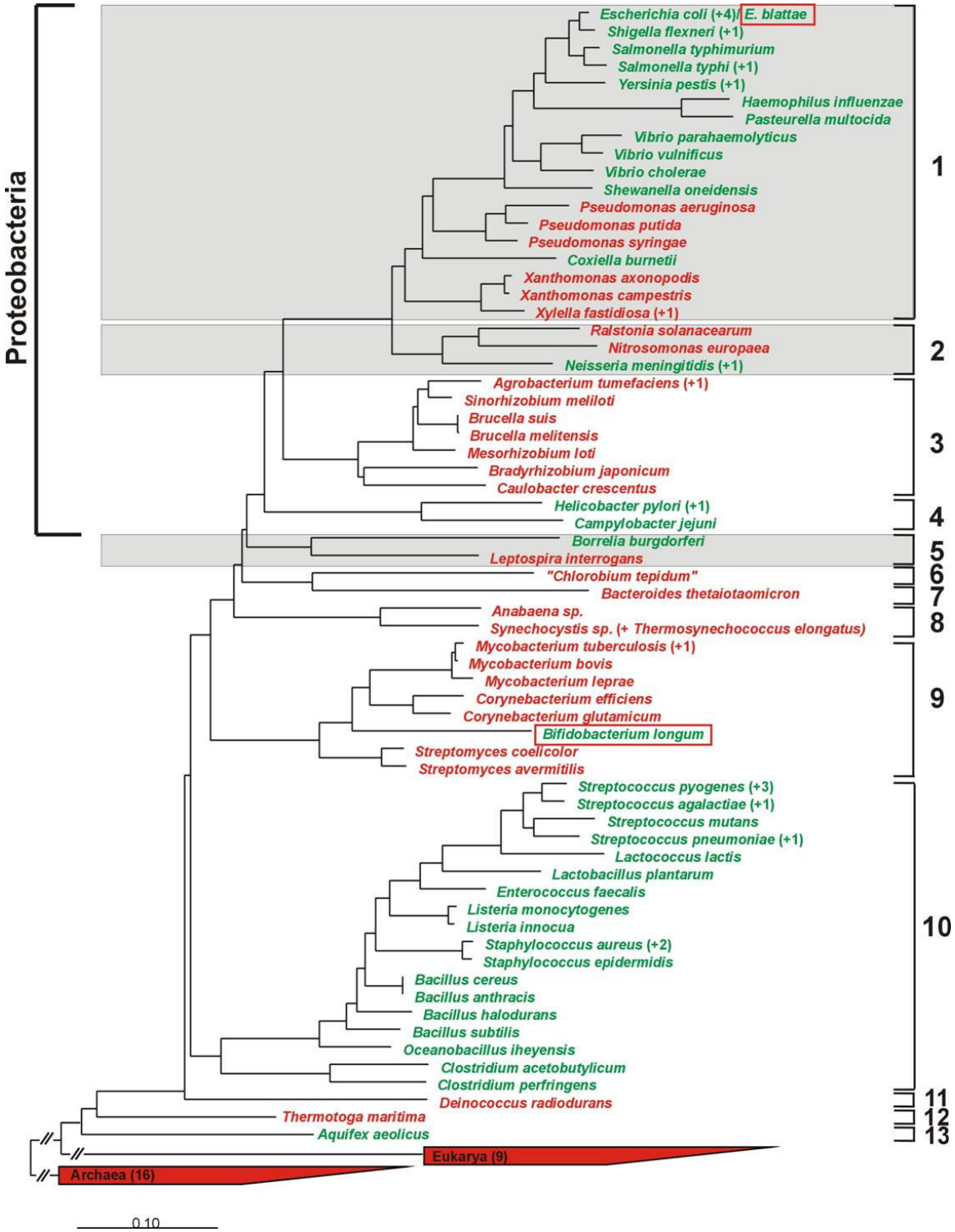


Figure 1-7. Phylogenetic distribution of the Pfs/LuxS and the SahH pathway in fully sequenced organisms (Sun *et al.*, 2004).

The figure illustrates a phylogenetic tree of the fully sequenced organisms and it is colour-coded to show which pathway is used in one organism. Bacteria written in green use the Pfs/LuxS pathway, while the others written in red use the SahH pathway. Boxed strains have both pathways. Numbers in brackets indicate sequenced genomes analysed. Phyla are numbered from 1 to 13. 1 *Gammaproteobacteria*; 2 *Betaproteobacteria*; 3 *Alphaproteobacteria*; 4 *Epsilonproteobacteria*; 5 *Spirochaetes*; 6 *Chlorobia*; 7 *Bacteroidetes*; 8 *Cyanobacteria*; 9 *Actinobacteria*; 10 *Firmicutes*; 11 *Deinococcus-Thermus*; 12 *Thermotogae*; 13 *Aquificae*.

1.2.6 AI-2 production and AI-2 activity

As AI-2 production is tightly linked to metabolism, two consequences can be deduced about its level. Firstly, the level of AI-2 of the same species will vary during growth, and also in different metabolism status and in different cultivating medium, as shown with *Salmonella typhimurium* (Surette & Bassler, 1999). Secondly, the level of AI-2 can vary within species and even within strains of the same species, since the activated methyl cycle is a part of a complex metabolic network and can be differentially important in bacterial species. For instance, the pathogenic serotype *S. typhimurium* 14028 produces significantly more AI-2 than the non-pathogenic *S. typhimurium* LT2 does (Surette *et al.*, 1999).

Therefore, if a species contains the *luxS* gene, there is still the question, at which level, in which growth stage does it produce AI-2? Is the AI-2 level medium-dependent? Do closely relative species with different life mode produce more AI-2?

1.2.7 The genus *Shewanella*

The genus *Shewanella* is a genus of the *Gammaproteobacteria*, whose members are represented worldwide, are versatile in their life mode and can adapt to even extreme niches (Hau & Gralnick, 2007). Species were found in the Antarctic (*Sh. frigidimarina*, *Sh. livingstonensis*) and in the tropical seas (*Sh. algae*), in freshwater (*Sh. oneidensis*) and in sea water (*Sh. gelidimarina*), as free living (*Sh. fidelis*) or associated with animals (*Sh. marinintestina, Sh. schlegeliana*) and also under high pressure (*Sh. violaceae*).

Some *Shewanella* species are even opportunistic pathogens. In clinical specimens, *Sh. algae*, *Sh. putrefaciens* and *Sh. oneidensis* (Venkateswaran *et al.*, 1999) were found. Most of the infections are probably caused by *Sh. algae*, which proved to be more virulent than *Sh. putrefaciens* (Holt *et al.*, 2005; Khashe & Janda, 1998; Vogel *et al.*, 2000). In the earlier times, *Shewanella* species were underestimated as potential pathogens, because they were always identified in mixed-species infections with known pathogens. However, in the last decade, single *Shewanella* infections were increasingly observed in a warm sea near to Australia (Skatssoon, 2004) and surprisingly also in the far colder Denmark (Gram *et al.*, 1999). In most of the cases, ear infection was diagnosed in children (Holt *et al.*, 1997). In addition, bacteremia and soft tissue and skin infections were manifested in elder patients having participated on long-term medication (Pagani *et al.*, 2003; Tsai & You, 2006).

However, none of the *Shewanella* species were investigated in concern of quorum sensing and their AI-2 production was also never shown, though the fully-sequenced species contain the *luxS* gene. If also the non-sequenced species are confirmed to have the *luxS*

gene, this genus can be a representative subject to investigate AI-2 production of closely related bacteria from different niches.

1.2.8 Shewanella oneidensis

Sh. oneidensis is the most studied and the first sequenced member in the genus *Shewanella* because of its unique versatility for anaerobic respiration (Heidelberg *et al.*, 2002).

It is capable of extracellular respiration with many different electron acceptors including Mn(III), Mn(IV), Fe(III), Cr(VI), U(VI)), fumarate, nitrate, trimethylamine *N*-oxide, dimethyl sulfoxide, sulphite, thiosulfate, and elemental sulphur. Such a high flexibility with electron acceptors is unique (Heidelberg *et al.*, 2002). The respiratory capabilities imply an array of possible biotechnological applications, for example, it might be useful in removing chlorinated compounds, radionuclides, and other environmental pollutants from wastewater. Also, the first attempts have been made to apply this strain as a biofuel cell, i.e. to use of its metabolism to generate electricity (Hau & Gralnick, 2007). Since some of the electron acceptors of respiration are solid, the bacteria could express higher respiratory effectiveness, it they attach to the surface and form biofilms. In accordance, *Sh. oneidensis* is well-known for developing structured biofilms (Thormann *et al.*, 2004)

The genome analyses of *Sh. oneidensis* revealed a few interesting facts about its virulence (Heidelberg *et al.*, 2002). It turned out that the genome of *Sh. oneidensis* is most similar to that of *Vibrio cholerae*, a well-known human pathogen and extensive regions of similar gene order were observed between the two bacteria. Since both reside in the sediment of rivers, they might have exchanged some of their genes. Furthermore, some virulence factors, like RTX toxin and type IV pili gene clusters, were identified. In addition, *Sh. oneidensis* has been already isolated from clinical specimens.

In summary, *Sh. oneidensis* has a number of respiratory capabilities, and thus it is extremely flexible and potentially useful both in biotechnological applications and in energy generation. It can form biofilms, possesses different pathogenic traits and its genome is highly similar to *V. cholerae*, a common human pathogen. Although, *Sh. oneidensis* contains the *luxS* gene, nothing is known about its quorum sensing system and its AI-2 production has also never been determined. Since AI-2 is a potential quorum sensing signal, it remains to be elucidated, which phenotypes might be regulated by AI-2 and what a role AI-2 could fulfil in this bacterium?

2 Aim of the work

AI-2 is a quorum sensing signal in *Vibrio harveyi*. It is produced by many other bacteria and could represent an interspecies-signalling molecule. A specific mechanism of uptake and processing of AI-2 was elucidated in *S. typhimurium* and *E. coli* and several quorum sensing-related phenotypes could be identified in mutants unable to produce AI-2, also named *luxS* mutants. This study is focused on the role and production of AI-2 in bacteria that have not been investigated so far. Specifically the following topics were covered:

1. **The regulatory role of AI-2 in *P. aeruginosa*.** *P. aeruginosa* does not produce AI-2, but it was shown to moderately upregulate a few of its quorum sensing-controlled virulence genes in the presence of AI-2 (Duan *et al.*, 2003). To elucidate if AI-2 is able to upregulate these genes and eventually other important genes, two of these reported genes were investigated with reporter plasmids in *P. aeruginosa* PAO strains.

2. **The phylogenetic distribution of the *luxS* and *sahH* genes in marine species.** In bacteria, there are two possible pathways for SAH recycling in the activated methyl cycle: the Pfs/LuxS pathway, by which AI-2 is produced, and the SahH way, by which it is not produced. As reported in a previous study (Sun et al., 2004), the presence of the pathways appears to be consistent within one phylogenetic group, and therefore the potential for AI-2 signalling of a species could be determined by the phylogenetic position, and independent of the life mode. However, mainly pathogenic species had been investigated. To confirm this hypothesis, 164 phylogenetically characterized marine isolates were investigated for the presence of the *luxS* and *sahH* genes representing these two pathways for SAH recycling.

3. **The AI-2 production in the genus *Shewanella*.** In the screening for the *luxS* gene, the *Shewanella* species were identified, as species which consistently exhibit the *luxS* gene. In addition, they are present worldwide in different niches. In order to clarify any correlation of AI-2 production with the niche of a species, the AI-2 production of ten *Shewanella* species was analysed in terms of level and pattern during growth.

4. **Phenotypes potentially regulated by AI-2 in *Sh. oneidensis*.** Within the *Shewanella* genus, the model organism and the first fully-sequenced member is *Sh. oneidensis*, and thus this species was chosen for analyses on the role of AI-2. Therefore, *luxS* knockout mutants were constructed and phenotypic comparisons were undertaken for growth, AI-2 production, expression of secreted proteins, biofilm formation and siderophore production.

3 Materials and Methods

3.1 Strains

Table 1-3. *Alishewanella fetalis* and *Shewanella* type strains.

Strain	No.	Reference	Cultivation
Alishewanella fetalis	CCUG 30811	Vogel *et al.*, 200)	LB, 37 °C
Shewanella algae	DSM. 9167	Nozue *et al.*, 1992;Simidu *et al.*, 1990	LB, 37°C
Shewanella fidelis	LMG 20552	Ivanova *et al.*, 2003	SM/MB, RT
Shewanella frigidimarina	LMG 18921	Bowman *et al.*, 1997	SM/MB, RT
Shewanella japonica	LMG 19691	Ivanova *et al.*, 2001	SM/MB, RT
Shewanella livingstonensis	LMG 19866	Bozal *et al.*, 2002	-*
Shewanella marinintestina	IK-1	Satomi *et al.*, 2003	SM/MB, RT
Shewanella oneidensis	MR-1	Venkateswaran *et al.*, 1999	LB, 30 °C
Shewanella sairae	SM2-1	Satomi *et al.*, 2003	SM/MB, RT
Shewanella schlegeliana	HRKA-1	Satomi *et al.*, 2003	SM/MB, RT
Shewanella violacea	LMG 19151	Nogi *et al.*, 1998	-*

* not cultivated, used only for PCR

** *A. fetalis* was obtained from Culture Collection, University of Goteborg. *Sh. algae* was purchased from the German Collection of Microorganisms and Cell cultures (DSM). *Sh. fidelis*, *Sh. frigidimarina*, *Sh. japonica*, *Sh. livingstonensis* and *Sh. violacea* were ordered from Belgian Co-ordinated Collections of Microorganisms (BCCM). *Sh. marinintestina*, *Sh. sairae* and *Sh. schlegeliana* were kindly sent by Masataka Satomi from National Institute of Fisheries Science, Japan. *Sh. oneidensis* was supplied as a gift by Joel Klappenbach from Michigan State University, Center for Microbial Ecology.

Table 1-4. *Escherichia coli* strains.

Strain	No.	Application/ Genotype	Source/ reference
E .coli	S17λ-*pir*	Biparental mating Tp^R Sm^R ,recA, thi, pro, hsdR-M+RP4: 2-Tc::Mu-Km::Tn7, λpir	Biomedal (Spain); Simon *et al.*, 1983
E. coli	*pir*-116	Maintaining of R6K *ori* plasmids F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL nupG pir-116(DHFR)	Epicentre; Metcalf *et al.*, 1994
E. coli	DH5α	Subcloning routine F- endAI hsdRJ7 (r-, mit) supE44 thi-J ArecAl gyrA96 relAI deoR A(lacZYA-argF)-U169480dlacZAM15	Invitrogen ; Grant *et al.*, 1990
E. coli	DH10B	Subcloning routine F- endAI hsdRJ7 (r-, mit) supE44 thi-J ArecAl gyrA96 relAI deoR A(lacZYA-argF)-U169 480dlacZAM15	Invitrogen ; Grant *et al.*, 1990
E. coli	HB101	Carrier of the pRK2013 plasmid for triparental mating. F^- mcrB mrr hsdS20(r_B^- m_B^-) recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20(Sm^R) glnV44 λ^-	Boyer & Roulland-Dussoix, 1969

Table 1-5. *Shewanella oneidensis genetically* modified constructs.

Strain	No.	Genotype/ description	Reference
Sh. oneidensis	WT_{Km}	Contains the pKnock-Km plasmid inserted before the *luxS* gene	this study
Sh. oneidensis	$luxS^-_{ins}$	Contains the pKnock-Km plasmid inserted in the middle of the *luxS* gene	this study
Sh. oneidensis	$luxS^-_{del}$	Lacks the *luxS* gene completely	this study

Table 1-6. *Vibrio harveyi* strains.

Strain	No.	Genotype/ description	Reference
Vibrio harveyi	BB152, BAA-1119	*luxM*::Tn5; produces only AI-2	Bassler *et al.*, 1993
Vibrio harveyi	BB170, BAA-1117	*luxN*::Tn; senses only AI-2	Bassler *et al.*, 1993
Vibrio harveyi	BB886	*luxP*::Tn; senses only AI-1	Bassler *et al.*, 1994
Vibrio harveyi	MM77	*luxM*::Tn5, *luxS*::CmR; produces neither AI-1 nor AI-2	Mok *et al.*, 2003

** *V. harveyi* BB152 and BB170 were purchased from the American Type Culture Collection. *V. harveyi* BB886 and MM77 were kindly sent by Bonnie L. Bassler, Princeton.

Table 1-7. *Pseudomonas aeruginosa* strains.

Strain	No.	Genotype/description	Reference
P. aeruginosa	$PAO1_{Igl}$	wildtype with Iglewski background *	Holloway *et al.*, 1979
P. aeruginosa	$PAO1_{O/V}$	wildtype with Ochsner/ Vasil background *	Holloway *et al.*, 1979
P. aeruginosa	PAO1-$MW1_{lasI^-, rhlI^-}$	Tc^R, $HgCl_2{}^R$; double *lasI*$^-$ and *rhlI*$^-$ mutant PAO1-$MW1_{Igl}$	Whiteley *et al.*, 1999
P. aeruginosa	PAO1 $_{lasR^-, rhlR^-}$	Tc^R, Gm^R; double *lasR*$^-$ and *rhlR*$^-$ mutant $PAO1_{Igl}$	Pearson *et al.*, 1997

* Iglewski background indicates modifications on the wildtype strain resulting from several subcultivations in the laboratory of Mrs Iglewski. The most apparent difference between the strains with Iglewski and with Ochsner/ Vasil background is their colour, while the strain with Iglewski background is yellow; the strain with Ochsner/ Vasil background is brownish. There was also discrepancy between their competences.
** All strains were kindly supplied by Martin Schuster from Oregon State University, Microbial Department.

3.2 Plasmids

Table 1-8. Plasmids for transformation into *Pseudomonas aeruginosa*.

Plasmid name	Antibiotic resistance and description	Genbank no.	Reference
pKR-C12	Sensor plasmid for 3-oxo-C12-HSL Gm^R; pBBR1MCS-5 carrying P_{lasB}-gfp(ASV) P_{lac}-*lasR*	n.a.	Riedel *et al.*, 2001
pMS402-*phzA1*	Km^R, pMS402 plasmid carrying the promoter region of the *phzA1* gene fused to *lux*CDABE reporter	n.a.	Duan *et al.*, 2003

n.a.: not available

Table 1-9. Plasmids for genetic modifications in *Shewanella oneidensis*.

Plasmid name	Application/ Description	Genbank no.	Source/ Reference
pKnock-Km	Km^R; *ori*T; source plasmid for the suicide vectors into *Sh. oneidensis*	n.e.	NCCB 3407; Alexeyev, 1999
pEX18Ap	Amp^R; source plasmid for a replicating plasmid into *Sh. oneidensis*	pEX18Gm: AF047518	Max Schobert, TU-Braunschweig; Hoang *et al.*, 1998
pPS858	Amp^R, Gm^R; source of the Gentamicin-GFP (Gm-GFP) cassette	n.e.	Max Schobert; Hoang *et al.*, 1998
pFLP2	Amp^R; FRT recombinase expression vector; source of the *sacB* gene	AF048702	Martin Schuster; Hoang *et al.*, 1998
pBSL97	Amp^R, Km^R; source of a Kanamycin cassette	XXU35136	NCCB 3384; Alexeyev *et al.*, 1995
pRK2013	Km^R; helper plasmid for conjugation	n.a.	DSMZ 5599; Figurski *et al.*, 1979
pKnock-*luxS*	For *Sh. oneidensis* $luxS^-_{ins}$ Km^R ; pKnock-Km containing an internal fragment of *luxS* of *Sh. oneidensis*	n.a.	This study

pBA2106	For *Sh. oneidensis* WT_{Km} Km^R , pKnock-Km contains a upstream fragment of *luxS* of *Sh. oneidensis*	n.a.	This study
pBA17106	Km^R, Gm^R; the pKnock-Km contains an upstream and a downstream fragment of *luxS* of *Sh. oneidensis* and, between them, the Gm-GFP cassette.	n.a.	This study
pBA1147	For *Sh. oneidensis* $luxS^-_{del}$ Km^R, Gm^R; *sacB* gene from pFLP2 ligated into pBA17106	n.a.	This study
pEX18ApGm	For green-fluorescing cells Km^R, Gm^R , the Gm-GFP cassette ligated into pEX18Ap	n.a.	This study
pFLP2-Km	For removing the Gm-GFP cassette FLP recombinase expression vector; pFLP2 ligated to the Km cassette of pBSL97	n.a.	This study

n.a.: not available

3.3 Primers

Table 1-10. Degenerated primers for the *luxS* and the *sahH* gene.

Primer name	Primer sequence	Anneal. temp.
LuxS_degfor3	CAT TAT TAG ATA GCT TTA CAD TNG AYC AYA	48 °C
LuxS_degrev4	AGC GAG TGC ATC TGA TAA GWN CCR CAY TS	48 °C
LuxS_gammafor1	TRG ATA GCT TTA CHG TTG ACC ATA C	48 °C
LuxS_gammarev2	TAR AAR CCN GTR CGA CAT CCC AT	48 °C
SahH_degfor	GAS GAS ACN ACN ACN GGN GT	46 °C
SahH_degrev	TCV DWR TCR AAR TGD CCR ATR TT	46 °C
SahH_alphafor	GCH GAR ACV GAR ATG CCS GGB YT	60 °C
SahH_alpharev	GAR CCY TTR CCS ACR TYR CCR WA	60 °C

Table 1-11. Primers for genetic manipulations in *Sh. oneidensis*.
Nucleotides in bold indicate the built-in restriction sites.

Primer name	Primer sequence	Anneal. temp.
CluxS_NotI_for[a]	TGG CAG AGA ACT GTT TAG **GCGGCCGC** AAC AGG CTC GCT TGA CG	66 °C
BluxS_BamHI_rev[a]	ATG GCA TAG AGA TCT CCA **GGATCC** CAG GGC GAT ACA ACG CCA C	66 °C
AluxS_BamHI_for[a]	TGC AAA TTC AGT TAT TTT **GGATCC** TAA CAA CAA ACG CCG TAC GC	66 °C
DluxS_KpnI_rev[a]	GCG CCA AGT AAG CTT GAA **GGTACC** ACC TTA AAC AAC ATA TCC AT	66 °C
LuxS_EcoRI_beg[a]	CAT TAC TTG **GAATTC** TTA CCG TTG ACC ATA CTC	60 °C
LuxS_KpnI_mid[a]	CAT TGG A **GGTACC** TC AAT AAT TTC CAC ATC	60 °C
pKnock_for[b]	GTG ACA CAG GAA CAC TTA AC	55 °C
pKnock_rev[b]	GAG TGC TTG CGG CAG CGT GA	55 °C
VluxSfor[b]	AGC AAC GGC AGC ATT AAT GCC A	60 °C
NluxSrev[b]	GTA GAA TGA TAC TGA TCA TTG A	60 °C
Sonfor[b]	TGGAGAGGTACTTAAGCGTGTTG	60 °C
Sonrev[b]	CAGTGATCCCAAGCAATGAA	60 °C
SonluxSfor[b]	TCG CCC TGA TAA TTT GGA GA	60 °C
SonluxSrev[b]	CAC GCC AGC TGC GAT AAT A	60 °C

[a] cloning primers were used with *Pwo* proofreading polymerase
[b] verification primers were used in standard PCR with *Taq* polymerase

Table 1-12. Primers for verification insert length in pMS402 plasmid.

Primer name	Primer sequence	Anneal. temp.
pZE.05	CCA GCT GGC AAT TCC GA	48 °C
pZE.06	AAT CAT CAC TTT CGG GAA	48 °C

3.4 Bacterial cultivation

3.4.1 Media

All media were solidified with 15 g/l agar and sterilized by autoclaving at 121 °C for 15 min.

3.4.1.1 Luria-Bertani Medium

25 g LB Miller (Sigma) and 40 g LB agar (Roth) was dissolved in 1 l deionised water for liquid and for solid LB respectively. These ready-to-use media consisted of the following components: 10 g/l tryptone, 5g/l yeast extract, 10g/l NaCl and in case of agar plates 15 g/l bacto agar.

3.4.1.2 Marine broth (MB)

Solid MB was used as a recovery medium after glycerol storage for marine *Shewanella* strains. Liquid MB was used for cultivating *Sh. hafniensis* DT-1 and *Sh. sairae*.

37.4 g/l ready-to-use MB broth (Difco) was dissolved in 1 l deionised water.

3.4.1.3 LB sea salt broth (LBSS)

Liquid LBSS was used for cultivating *Sh. hafniensis* DT-1.

25 g/l LB Miller (Sigma) and 17 g/l sea salt (Sigma) were dissolved in deionised water and the solution was autoclaved at 121 °C for 15 min.

3.4.1.4 *Shewanella* marine medium (SM)
(Makemson *et al.*, 1997)

The medium was used in liquid form for cultivating the marine *Shewanella* strains.

5 g bacto peptone (Difco) and 1 g yeast extract (Difco) were dissolved in about 700 ml deionised water. This solution was amended with 200 ml 5x salt solution and filled up to 1 litre. The pH was adjusted to 7 with NaOH.

5x salt solution:

150 g	NaCl
25.5 g	$MgCl_2$-6-hydrat
30.8 g	$MgSO_4$-7-hydrat
7.5 g	KCl

3.4.1.5 *Shewanella* Defined Minimal medium (SDM)

(Myers & Nealson, 1988)

Solid SDM was used for plating the conjugation mix and for the siderophore test. Liquid SDM was used for the biofilm monitoring.

SDM basic:

100 ml	salt solution
10 ml	iron solution
10 ml	trace metal solution
3.53 ml	Na-D,L-lactate(Fluka, 50%) (i.e. 20mM final concentration)

The SDM basic solution was adjusted to pH=7.5 with NaOH and filled up to 970 ml with deionised water.

After cooling down the following solutions were added to SDM basic:

10 ml	phosphate buffer
10 ml	$NaHCO_3$ solution
10 ml	amino acid solution

Salt solution (10x concentrated) per litre:

2.46 g	$MgSO_4$-7-hydrat
11.9 g	$(NH_4)_2SO_4$
0.7 g	$CaCl_2$

It was stored at 4°C until use.

Iron solution (100x concentrated) per litre:

0.15 g	$FeSO_4$
2.5 g	EDTA

It was stored at 4°C until use.

Trace metal solution (100x concentrated) per litre:

0.35 g	H_3BO_3
0.06 g	NaCl
0,14 g	$CoSO_4$-7-hydrat
0.2 g	$Ni(NH_4)_2(SO_4)_2$
0,03 g	$ZnSO_4$-7-hydrat
0.005 g	$CuSO_4$-5-hydrat
0.09 g	Na_2MoO_4-2-hydrat
0.03 g	Na_2SeO_4
0.02 g	$MnSO_4$

It was stored at 4°C until use.

Phosphate buffer (100x concentrated), per litre:

99.3 g	K_2HPO_4
44.9 g	KH_2PO_4

It was sterilized by filtration through a 0.22 µm membrane and stored at 4°C until use.

$NaHCO_3$ solution (100x concentrated), per litre:
16.8 g $NaHCO_3$
It was freshly prepared and sterilized by filtration through a 0.22 µm membrane.

Amino acid solution (100x concentrated), per litre:
20 µg/ml L-arginine (Aldrich)
20 µg/ml Na-L-glutamate (Merck)
20 µg/ml DL-serine (Merck)
It was sterilized by filtration through a 0.22 µm membrane and stored at 4°C until use.

3.4.1.6 Autoinducer bioassay medium (AB)
(Greenberg *et al.*, 1979;Vilchez *et al.*, 2007)

It was used in solid and liquid form for cultivating *V. harveyi* strains.

AB basic:
17.5 g NaCl
12.3 g MgSO4-7-hydrat

This solution was filled up to 940 ml with deionised water.

After cooling down, the following solutions were added from sterile stocks:
10 ml 1 M potassium phosphate buffer (pH=7.0)
10 ml 0.1 M L-arginine
20ml 50 % glycerol
20ml 10 % Casamino acid (Difco)

The potassium phosphate buffer was mixed from 1 M K_2HPO_4 and 1 M KH_2PO_4 until pH=7 was reached.

Liquid AB medium was additionally supplemented directly before inoculation with a sterile solution containing trace elements and vitamins (Vilchez *et al.*, 2007). This solution contained the following components: 7.4 µM KI; 19 mM $MgCl_2$-hydrat; 255 µM $ZnCl_2$; 694 µM $FeCl_3$-6-hydrat; 43 µM $CuCl_2$-2-hydrat; 118 µM $MnCl_2$-4-hydrat; 10 µM $(NH_4)_6Mo_7O_{24}$-4-hydrat; 110 mM $CaCl_2$-hydrat; 2 µM Na_2SeO_4; 30 µM $CrCl_3$-6-H_2O; 23 µM retinol; 14 µM β-carotene; 0.13 µM cholecalciferol; 0.7 µM vitamin K1; 0.2 µM (+)-α-tocopherol; 0.04 µM thiamine hydrochloride; 0.04 µM (–)-riboflavin; 0.1 µM pyridoxine hydrochloride; 0.007 µM vitamin B12; 1.5 µM nicotinic acid; 0.3 µM pantothenicacid hemicalcium salt; 4.5 µM folic acid; 0.6 µM, biotin and 340 µM L-ascorbic acid.

3.4.1.7 SOC medium

(Sambrook & Maniatis, 2001)

This medium was used to recover the *E. coli* cells after transformation. It is mixed from SOB medium and glucose.

SOB medium

20 g	Tryptone (Difco)
5 g	Yeast Extract (Difco)
0.5 g	NaCl (Roth)
10 ml	0.25 M KCl
5 ml	2 M $MgCl_2$

The components were dissolved in 980 ml deionised water, and the pH was adjusted to 7.

For one litre SOC medium, 20 ml 1 M sterile D-glucose (Sigma) was added to SOB medium.

3.4.2 Antibiotics

Antibiotic stocks of ampicillin (Amp), gentamicin (Gm) and kanamycin (Km) were prepared at 10 or 100 mg/ml concentrations in deionised water and sterilized by filtration through a 0.22 µm membrane. Tetracycline (Tc) stock was dissolved in methanol at 10 mg/ml concentration. All were stored as 1 ml aliquots at -20 °C until use. $HgCl_2$ was dissolved in water at 4 mg/ml concentration and stored at 4 °C.

E. coli strains were selected with Amp at 150 µg/ml, Km at 50 µg/ml, Gm at 20 µg/ml and Tc at 25 µg/ml concentration respectively. *Sh. oneidensis* was selected with Gm at 20 µg/ml and Km at 100 and 200 µg/ml concentration after conjugation and during cultivation respectively. *P. aeruginosa* PAO1-MW1$_{lasI^-,\ rhlI^-}$ was selected with Tc at 50 µg/ml and $HgCl_2$ at 15 and 7.5 µg/ml concentration in solid and liquid medium respectively. *P. aeruginosa* PAO1 $_{lasR^-,\ rhlR^-}$ was selected with Tc at 50 µg/ml and Gm at 300 µg/ml concentrations. Km at 700 µg/ml concentration was used to select for pKR-C12 containing cells.

3.4.3 Cultivation conditions

All bacteria were cultivated aerobically using standard microbiological techniques. They were streaked onto an agar plate from a glycerol stock. The colonies which appeared were inoculated into 20 ml liquid medium in a 100 ml Erlenmeyer flask. This pre-culture grew overnight. The next morning, the main culture was inoculated from the pre-culture to 1 % density. All cultures were being shaken at 160 rpm on a rotary shaker. Growth was

monitored by measuring optical density at 600 nm (OD_{600nm}) with a Pharmacia Biotech spectrophotometer.

The species differed, in their temperature optima and oxygen demand. *E. coli* and *P. aeruginosa* strains grew at 37 °C in LB in baffled flasks. *V. harveyi* was cultivated at 30 °C in AB in baffled flasks. *Sh. oneidensis* was cultivated at 30°C in LB in non-baffled flasks. This bacterium grew very well at RT too, but this temperature was rarely used and will be mentioned with the concerned experiment. Other *Shewanella* species were grown as indicated in Table 1-3 and in non-baffled flasks.

Culture supernatants were prepared hourly during the first day of incubation, and a few times on the second day of incubation.

3.4.4 Glycerol stock

E. coli and *V. harveyi* strains were stored with 15 %, *Shewanella* strains with 10 % or 15 % glycerol.

The glycerol stocks were prepared from overnight cultures, or, in rare cases, from OD_{600nm}=1 cultures. For stock preparation, 1.5 ml culture was centrifuged in a cryotube at 5000 x g for 5 minutes at RT. The supernatant was decanted; the pellet was dissolved in 500 µl LB with glycerol. The stock was gradually chilled to -70 °C: first, it was held on ice for 30 minutes, and then at -20 °C for a couple of hours, finally it was brought to the -70 °C freezer.

3.5 Design of degenerated primers

The most conserved regions were chosen on the basis of multiple alignments (ClustalW) of LuxS and SahH amino acid sequences. The alignment of the corresponding nucleic acid sequences was used to assemble the sequence of the oligonucleotide. All primers are listed in Table 1-10.

The *luxS* gene is about 500 bp long and seemed only moderately conserved on the basis of alignment of 24 *luxS* genes of Gram negative bacteria. To address this problem, the consensus-degenerate hybrid oligonucleotide primer design strategy (CoDeHOP) (Rose *et al.*, 1998) was applied. According to this approach, the primers are composed of two parts: a highly degenerated 5' and a specific 3' end. The function of the degenerated 5' end was to bind to all possible templates in the first PCR cycles. Therefore, it targeted a region of 3-4 highly conserved amino acids and covered all possible variations present in the nucleic acid sequence alignment. The role of the specific 3' end was to stabilize the binding of the

primers in the latter PCR cycles. The 3' end was about 20 bp long and constituted of the most frequently occurring nucleic acid at each position. The created oligonucleotides were LuxS_degfor3 and LuxS_degrev4 primers and amplified approximately 400 bp of the *luxS* gene. In addition, a second, group-specific, primer set was designed for the *luxS* gene in *Gammaproteobacteria* without the CoDeHOP approach. The LuxS_gammafor1 primer targeted the same region as LuxS_degfor3. The LuxS_gammarev2 primer targeted the highly conserved mGC*TG*y region in the middle of the *luxS* gene. A fragment of 250 bp was amplified with this primer set.

The *sahH* gene is highly conserved and about 1400 bp long. It was not necessary to use the CoDeHOP strategy. On the basis of alignment of 60 bacterial *sahH* sequences, a universal primer set, SahH_degfor and SahH_degrev, was designed. They amplified 470 bp. For a few *Alphaproteobacteria*, if no PCR product was detected, another group-specific primer pair, SahH_alphafor and SahH_alpharev, was used. SahH_alphafor targeted the AETEMPGL region that is completely identical in all sequenced *sahH* genes in *Alphaproteobacteria*. The primer SahH_alpharev targeted the G*G*VGKGS conserved region around the middle of the gene which is a part of the NAD binding domain (Sganga *et al.*, 1992). They amplified about 700 bp.

3.6 Bioassays

3.6.1 The *Vibrio harveyi* bioassay

To detect AI-2 in the culture supernatant of the tested strains, the sensor strain, *V. harveyi* BB170, was used. It has a single defect in AI-1 sensing, and thus it can detect only AI-2.

The bioassay was performed as follows (Vilchez *et al.*, 2007). The sensor strain *V. harveyi* BB170 was cultivated as described above. Preinoculum cultures with a final OD_{600} of 1.0–1.1 were diluted 1:5000 time in AB, the working solution. Twenty microliters of the test samples, reference media and controls were transferred onto white microtitre plates (NUNC, Roskilde, Denmark), followed by the addition of 180 µl of working solution of the sensor strain. Four replicates were measured for every sample and six replicas for each control. Microtitre plates were incubated at 30 °C with agitation at 650 rpm and measured hourly for at least 6 h. Luminescence was measured hourly in a Victor Wallac Luminescence Reader.

Sterile culture supernatants from *V. harveyi* BB152 and MM77 served as positive and negative controls, respectively. Chemically synthesized AI-2 (De Keersmaecker *et al.*,

2005;Semmelhack *et al.*, 2005) diluted in AB medium was used as an additional positive control at a concentration of 5.3 µM.

The results presented as fold induction were obtained by dividing the luminescence of the samples by the luminescence of the sterile reference medium (Figure 1-8). The maximum of fold change was referred to as AI-2 activity. The AI-2 activity of the samples was compared to the AI-2 activity of the positive control, and this was the relative AI-2 activity.

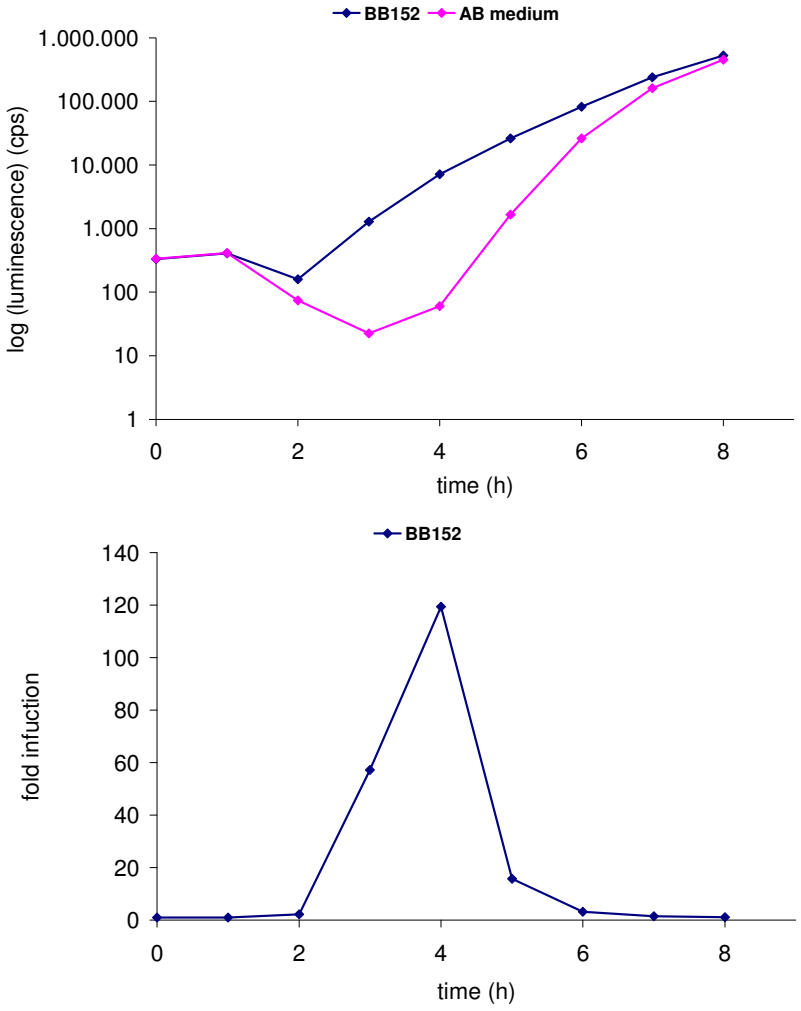


Figure 1-8. Calculation of AI-2 activity in the *Vibrio harveyi* bioassay.
In the bioassay, luminescence of the *Vibrio harveyi* sensor strain is measured. The luminescence in the wells with a reference medium (sterile AB medium) decreased and began to increase first after four hours of incubation. The luminescence with AI-2 containing culture supernatant (*V. harveyi* BB152) remained steady for two hours, and then increased. The two luminescence values were divided thereby providing the fold change of luminescence, also named AI-2 activity. AI-2 activity was usually maximal after 3 to 5 hours of the incubation.

3.6.2 Bioassays with *Pseudomonas aeruginosa* strains

This bioassay was used to test the effect of AI-2 on *P. aeruginosa* promoters. The sensor strains were *P. aeruginosa* PAO1-MW1$_{lasI^-, rhlI^-}$ with pRK-C12, GFP-reporter plasmid, and *P. aeruginosa* PAO1$_{lgl}$ strain with pRK-C12 plasmid and pMS402-*pHZ*A1, luminescence-reporter plasmid, respectively.

The sensor strains were pre-cultivated at 37 °C in LB overnight. The overnight culture was diluted 1:10, which was the working solution. A hundred microliter of the samples, reference media and controls were transferred into microtitre transparent plates (NUNC, Roskilde, Denmark), followed by the addition of 100 µl of working solution of the sensor strain. All samples, references and the positive control were in four replicas on the microtitre plates. The microtitre plates were incubated at 37 °C without shaking. In case of the pRK-C12 reporter plasmid, $GFP_{485/535nm}$ and OD was measured in transparent microtitre plates 0, 4, 8 and 24 h after inoculation. In the case of the pMS-*pHZ*A1 reporter plasmid, luminescence was measured in white microtitre plates hourly for at least 10-12 h and on the next day after 24 h.

The samples were the culture supernatant of *V. harveyi* BB152 containing AI-2, the culture supernatant of *V. harveyi* MM77 lacking AI-2 and DPD solutions at 53 µM and 5.3 µM. The positive control was 90 µl medium with 10 µl 3-oxo-C12-HSL dissolved in methanol at 0.1 mg/ml concentration. The references were AB medium for the culture supernatant of *V. harveyi*, LB medium for DPD solutions and 90 µl LB medium with 10 µl methanol for the positive control.

For data analysis, fold inductions were calculated. In the case of the GFP-reporter plasmid, first specific fluorescence was calculated by the dividing of GFP by OD. Then, fold induction was calculated by dividing the specific fluorescence of the sample by that of the reference. In the case of the luminescence reporter plasmid, fold induction was calculated by dividing the luminescence of the sample by that of the reference.

3.7 Standard DNA techniques

All enzymatic reactions were performed in 0.2 ml PCR soft tubes in an Eppendorf thermocycler. The bands were separated on agarose gels (NEEO ultra-quality agarose, Roth). If the expected length was longer than 1 kbp, 0.8 % agarose gels, if they were shorter, 1.5 % agarose gels were used. The bands were visualized by staining in 2 µg/ml ethidium bromide bath for 15-30 min.

3.7.1 Isolation of genomic and plasmid DNA

Genomic DNA and plasmid DNA were prepared by the Nucleospin Tissue kit (Macherey & Nagel) and by plasmid miniprep kits (Qiagen, Roche, Macherey Nagel, Omniscience) respectively according to the instructions of the manufacturer. The culture volume for plasmid DNA isolation ranged from 3 ml to 20 ml depending on the required concentration.

To verify clones containing modifications in the genomic DNA or at the plasmid DNA, lysates of single colonies were applied instead of pure DNA solutions. For this, a colony was picked out and dissolved in 20 µl MilliQ water in an Eppendorf tube. The tube was put at 95 °C for 20 minutes in a Thermoblock, to break the cells. Then the lysed cells were centrifuged at 17.000 x g for 1 minutes and the supernatant was applied in PCR reactions.

3.7.2 PCR

3.7.2.1 Standard PCR

Standard PCR with *Taq* polymerase was applied to screen for a specific PCR product of *luxS* or *sahH* genes, to verify a DNA or to sequence PCR products.

Taq polymerase, 10 x buffer and $MgCl_2$, were purchased ready-to-use from Qiagen. Lyophilized primers were delivered by Gibco or by Operon and were dissolved in water to 0.1 mM concentration. This stock was then diluted 1:10 to get 10 µM working solution. dNTPs were mixed from dATP, dCTP, dGTP and dTTP (Fermentas) at final concentration of 10 mM. MilliQ ultrapure water was obtained from a Millipore Water Purification system, autoclaved at 121 °C for 20 min and aliquoted in 1 ml portions. All components were stored at -20°C until use.

The PCR reaction was prepared in a master mix for all sample DNAs, for the positive and the negative control. The master mix contained the additives at the following concentrations: 1x PCR buffer, 3 mM $MgCl_2$, 200 µM dNTPs, 0.25 U/µl Taq, 1 µM forward and 1 µM reverse primer of each. This mix was portioned into PCR soft tubes and 1 µl of DNA solutions were added. A single PCR reaction had 20 µl or 50 µl final volumes. The thermocycler protocol was accomplished in an Eppendorf Mastercycler including the following steps: pre-denaturation at 94°C for 1 min, then 30 cycles of denaturation at 94°C for 10 sec, annealing for 30 sec at the temperatures given in Table 1-10, Table 1-11 and Table 1-12, elongation at 68°C, finally post-elongation at 68°C for 1 min 30 sec or for 10 min in case of longer PCR products. To screen for the *luxS* and *sahH*

genes, the elongation times were 40 sec and 50 sec respectively. In other cases, 1 minute elongation time was calculated for 1 kbp expected length of the PCR product.

3.7.2.2 High-fidelity PCR

For cloning PCR products, high-fidelity PCR with *Pwo* DNA polymerase was applied.

The polymerase and buffer containing $MgS0_4$ were obtained from Roche. Primers, dNTPs and MilliQ water were prepared as mentioned above (3.7.2.1)

A single PCR reaction had a final volume of 50 µl and was mixed from two master solutions. The first mix contained 600 nM of forward and 600 nM of reverse primer, 200 µM dNTPs, 1 µl DNA and MilliQ water up to 25 µl for each single reaction. The second mix consisted of 1x buffer, 2.5 U Pwo DNA polymerase and MilliQ up to 25 µl for each single reaction.

The thermocycler protocol included the following steps: pre-denaturation at 94°C for 2 min, then 10 cycles of denaturation at 94°C for 15 sec, annealing for 30 sec at the temperatures given in Table 1-11, elongation at 72°C for times given next, and then 20 cycles of denaturation at 94°C for 15 sec, annealing for 30 sec, elongation at 72°C for times given next and in each cycle for an additional 5 sec more, and finally post-elongation at 72°C for 7 min. The elongation time of *luxS1* was 30 sec. Elongation times of the upstream and downstream fragments of *luxS* were both 1 min.

The PCR products were purified by PCR purification kits from diverse companies (Qiagen, Macherey Nagel, Omniscience).

3.7.3 Restriction digestion

Restriction digestion was used for two subsequent purposes: ligation and verification.

The highly concentrated (50 U/µl) restriction enzymes with their buffers were purchased from Fermentas. The reactions were performed at 37 °C (optimal temperature for all used enzymes) for 1 h.

For ligation, 6 mg of DNA (plasmid or purified PCR product), the recommended buffer, MilliQ water and the enzyme were mixed up to 40 µl final volume. The amount of enzyme was calculated according to 10 and 30 time overdigestion of the plasmid and the PCR product respectively. Unless the digested products were dephosphorylated afterwards, they were purified by PCR purification or gel extraction kits.

For verification, 5 µl plasmid DNA, the recommended buffer, 1 µl enzyme and MilliQ water were mixed up to 20 µl final volume.

3.7.4 DNA blunting

The pBA17106 plasmid and the *sacB* cassette were blunted using the T4 DNA polymerase (NEB) according to the instructions of the manufacturer, and then purified by PCR purification kit.

3.7.5 DNA dephosphorylation and phosphorylation

The inserts were always dephosphorylated. Only the *sacB* containing insert before blunt-cloning was phosphorylated.

For dephosphorylation, shrimp alkaline phosphatase (SAP, Fermentas) was used; for phosphorylation polynucleotide kinase (PNK, NEB) was used. Both enzymes were used according to the instructions of the manufacturer.

As a rule-of-thumb, one unit enzyme was calculated for 1 pmol DNA ends.

SAP was usually added directly to the restriction digestion mix together with the buffer. The mix was incubated at 37 °C for 30 min.

PNK and its buffer were added to the filled-up and purified *sacB* containing insert. The mix was incubated at 37 °C for 20 min.

The products were purified by PCR purification kits.

3.7.6 Ligation

Ligation kits were obtained from Epicentre or Fermentas (Quick Ligase).

The ligation mix had the following components: buffer, ATP, DNA including insert and plasmid; and MilliQ water up to a final volume of 15 µl. The reactions were carried out at 22 or 24 °C depending on the instructions of the manufacturer, for 10-20 minutes.

The total amount of DNA was 150-200 ng. The concentration of the insert and the plasmid was estimated before ligation on 0.8 % agarose gels using MassLadder (Fermentas). If the concentration was too low, the DNA was concentrated in Microcon YM-100 filters. The molar ratio of insert and plasmid was 3:1. The molar weight of the DNA is considered to be in linear correlation to its length. The exact molecular weight of a DNA was not required information for this aim.

After ligation, the whole mix was first purified by a PCR purification kit, and the purified mix was used for transformation into *E. coli*.

3.7.7 Sequencing

PCR products of the correct length were sequenced for confirmation.

The PCR band of interest was purified using a gel extraction kit (Qiagen, Macherey Nagel, Omniscience). The purified DNA was sequenced using the BigDye Terminator v3.1 Cycle 410 Sequencing Kit on an ABI 3730xl DNA analyser.

The forward and reverse sequences were analysed by NCBI Blast search and assembled with Vector NTI software.

3.8 DNA introduction into bacterial cells

3.8.1 Transformation of *Escherichia coli* by electroporation

Several methods exist for transformation of *E. coli*, but electroporation proved to be the most efficient one (Erik Pollman, personal communications).

To prepare electrocompetent cells, the bacteria were first allowed to grow in LB till OD_{600nm}=0.4. The culture was transferred into 50-ml Falcon tubes pre-chilled on ice. Five washing steps followed whereby the cells were pelleted by centrifugation at 4,000 x g at 4 °C for 15 min, and then the supernatant was decanted and the pellet was resuspended in pre-chilled MilliQ water or 10 % glycerol. The first time, the pellet was resuspended in the same amount of water as the original culture volume, the second time in half the volume of water, the third time in 1/10 amount of glycerol, the fourth time in 1/50 amount of glycerol, and finally, the fifth time in 1/500 volume of glycerol. The cell suspension was portioned in 40 µl aliquots in pre-chilled 1.5 ml Eppendorf tubes and placed immediately into – 70 °C liquid nitrogen. The competent cells were stored at -70 °C until use.

For electroporation, the competent cells were first thawed on ice. Then, 5 µl of plasmid DNA or purified ligation solution were pipetted to the cells, and the mix was transferred into 0.1-cm pre-chilled electroporation cuvettes (BioRad). Electroshock was applied at 18 kV/cm voltage, 200 Ω resistance and 25 µF capacitance in a BioRad Gene Pulser with Pulse Controller. Immediately after electroshock, 1 ml SOC medium was added to the cells. The cell culture was transferred into 15-ml Falcon tubes and incubated at 37 °C for 1-1.5 h with agitation, to allow the cells to express the antibiotic resistance. From the

recovered cells, serial dilution was prepared and 100 µl of the 10^0, 10^{-2} and 10^{-4} dilutions were spread on antibiotic LB plates.

The colonies which appeared on the next day were picked out and streaked onto a master plate for analysis by standard PCR (3.7.2.1). A few of the right clones were also confirmed by restriction digestion (3.7.3).

3.8.2 *Shewanella oneidensis* conjugation

Conjugation proved to be the only method to introduce DNA into *Shewanella oneidensis*. In the first experiments, biparental mating, i.e. mating with a donor and recipient strain, was employed, but in the later experiments, triparental mating, i.e. mating with a donor, a recipient and a helper strain, was used, because it was more efficient.

3.8.2.1 Biparental mating

The donor was *E. coli* S17-1 λ*pir* with the pKnock-*luxS1* or pBA2106 plasmid respectively. This strain has the *tra* genes on the genome which was responsible for conjugation. The recipient was the *Sh. oneidensis* wild-type strain.

Both the donor and the recipient strain were cultivated in LB till OD_{600nm}=1, which corresponds to about 10^8 cells/ml. Then, 1.5 ml of each culture was transferred into 2-ml Eppendorf tubes. The cells were washed twice by centrifugation (at 4.000 x g for 3 minutes at room temperature) followed by decanting the supernatant and resuspending the pellet in 1 ml LB. After one more centrifugation step, the supernatants were completely removed, and the pellets were dissolved in 30 µl LB and mixed. This mix was spotted onto mating disks, e.g. LB agar plates with a sterile mixed cellulose ester membrane (Millipore, MF type without triton) on the top. The bacteria on the mating disks were incubated at RT for 1 day. Then, the cells were resuspended in 1 ml PBS buffer at RT, and 200-300 µl of the suspension was plated onto SDM agar with Km 100. SDM medium served as amino acid auxotrophic *E. coli* S17-1, Km selected for the right *Sh. oneidensis* recombinants.

The colonies appeared after 1-2 weeks, and were picked out and streaked further to obtain pure colonies. The pure colonies were analysed by standard PCR.

3.8.2.2 Triparental mating

In triparental mating, the donor and the conjugation functions were fulfilled by two different strains. The donor strain was an *E. coli* strain, which optimally maintained the mobilizable plasmid. The conjugating strain was *E. coli* HB101 with the pRK2013, helper plasmid, which expressed pili required for conjugation from the pRK2013 plasmid.

Triparental mating was applied when pEX18ApGm plasmid from *E. coli* DH10B, pBA1147 plasmid from *E. coli pir*-116 and pFPLP2-Km plasmid from *E. coli* DH10B was conjugated into *Sh. oneidensis*. The conjugation protocol including pre-cultivation in LB, washing in LB, spotting of the cells onto mating disks, incubation and resuspension in PBS, was identical to the biparental mating protocol (3.8.2.1). After each conjugation, SDM agar served as a counter selection against *E. coli* cells due to amino acid auxotrophy. To select for *Sh. oneidensis* cells harbouring pEX18ApGm and pBA1147, SDM was complemented with Gm 20. To select for pFLP2-Km containing *Sh. oneidensis* cells, SDM was amended with Km 100.

The exconjugants appeared after 1-2 weeks, the *Sh. oneidensis* clones were first purified by subcultivation and then analysed by standard PCR.

3.8.3 Transformation of *Pseudomonas aeruginosa*

There are both chemical and electro-transformation methods for the introduction of DNA into *P. aeruginosa*. The strains with the "Iglewski background" could be transformed also by chemical transformation; the others with the "Ochsner/ Vasil" background could be transformed only by electroporation. In this study, the "Iglewski background" strains were tested in bioassays, therefore the method of chemical transformation (Chuanchuen *et al.*, 2002) will be detailed.

To obtain competent cells, the strains were cultivated at 37 °C in LB overnight. 1 ml of the stationary phase culture was transferred into a 1.5-ml pre-chilled Eppendorf tube and centrifuged at 17.000 x g for 30 seconds at RT. The supernatant was decanted; the pellet was resuspended in 1 ml ice-cold 0.1 M $MgCl_2$ and centrifuged again. Then, the supernatant was decanted, and the pellet was resuspended in 1 ml ice-cold TG salts (75 mM $CaCl_2$, 6 mM $MgCl_2$, 15 % glycerol) and kept on ice for 10 minutes. After one more centrifugation step and removal of the supernatant, the competent cells were dissolved in 200 µl TG salts.

For transformation, 5 µl of plasmid was added to 100 µl competent cells. The mix was kept on ice for 15 min, and then placed into a thermoblock at 37 °C for 1 min. Immediately after

this heat-shock, the cells were amended with 500 µl LB at RT. The culture was incubated at 37 °C for 1 h with agitation to recover the cells and to express the antibiotic resistance encoded on the plasmid. Finally, different volumes of the cell suspension were plated onto LB agar with antibiotics. Colonies appeared after 1 day and were patched onto a master plate. The clones were analysed by standard PCR or restriction digestion.

3.9 The *sacB* counter selection

This selection (Gao *et al.*, 2006) was used to force *Sh. oneidensis* strains that had integrated the pBA1147 plasmid in the genome to excize the plasmid by homologous recombination, and also to eliminate the pFLP2-Km plasmid from the *Sh. oneidensis* $luxS^-_{del}$ deletion mutant.

A single colony was inoculated into LB medium, which did not contain NaCl. After overnight incubation, the culture was serially diluted, and 200 µl of the 10^0, 10^{-2} and 10^{-4} dilutions were plated onto LB agar with 7 % sucrose and antibiotic if necessary. The clones appeared after a few days and were analysed by standard PCR.

3.10 Biofilm cultivation

Biofilms of *Sh. oneidensis* $luxS^-_{ins}$ and WT_{Km} with pEX18ApGm plasmid were grown on glass slides in Petri dishes. To maintain the plasmid insert in the genome and the pEX18ApGm in the cytoplasm, Km and Gm were added to the medium.

The strains were pre-cultivated at 30 °C in LB. The overnight culture was inoculated into the LB main culture to 2.5 % density to facilitate faster growth. The culture reached OD_{600nm}=0.7-1 after 4 hours.

In the pre-experiments (Figure 1-9), 12 ml of the LB cultures were pipetted onto the sterile glass slides (10 slides) in Petri dishes. After 45 min incubation at 30 °C, the slides were transferred into fresh 12 ml SDM medium and biofilm growth was monitored.

In the supplementation experiment (Figure 1-9), this pre-incubation step was eliminated. The LB cultures were directly inoculated into fresh SDM medium to 5 % density. These cell suspensions were divided into three parts. The first part remained untreated, the second and third parts were supplemented with 3.8 µM DPD and 1 mM L-methionine (Sigma) respectively. 12 ml of these cell suspensions were pipetted onto sterile glass slides (10 slides) and then biofilm growth was monitored.

These biofilm dishes were incubated at 30 °C. At each time point, one dish was removed for sample preparation. The glass slide was removed from the dish, and briefly rinsed in sterile PBS buffer. Then, the glass slide was covered with a glass slip and sealed with nail polish.

The biofilm structures on the slides were documented using a fluorescence microscope and a confocal laser scanning microscope. The fluorescence microscope were an Olympus BX60 microscope equipped with Plan objectives (Olympus, 40x, 0.65; 10x, 0.25) and with a fluorescence lamp (Olympus, model U-ULS100Hg), for which the light source was provided by a 100 W, high pressure mercury burner (Olympus, model BH2-RFL-T3). The imaging with the fluorescence microscope was performed with a digital camera (Colorview) fixed on the micrococope and transferred on PC with the aid of cellA imaging software. The confocal laser scanning microscope (CLSM) was a Fluoview 1000 (Olympus) microscope equipped with UPlanSApo objectives and simultaneously detected bright field and EGFP images (20 mW 561 nm solid state laser and 30mW 488 nm multiline argon laser with 2 % power respectively). The monochrome sequences of images of the CLSM microscope were measured along the optical axis with 1 µm increments. The Imaris X64 5.7.2 software was used for processing the images from the CLSM microscope and for estimating the biofilm thickness. Other biofilm parameters, as biomass and substrate coverage, were calculated by the Phlip Matlab toolbox (Mueller *et al.*, 2006) In the first experiment, a magnification of 40x, in the supplementation experiment, a magnification of 10x was usually used.

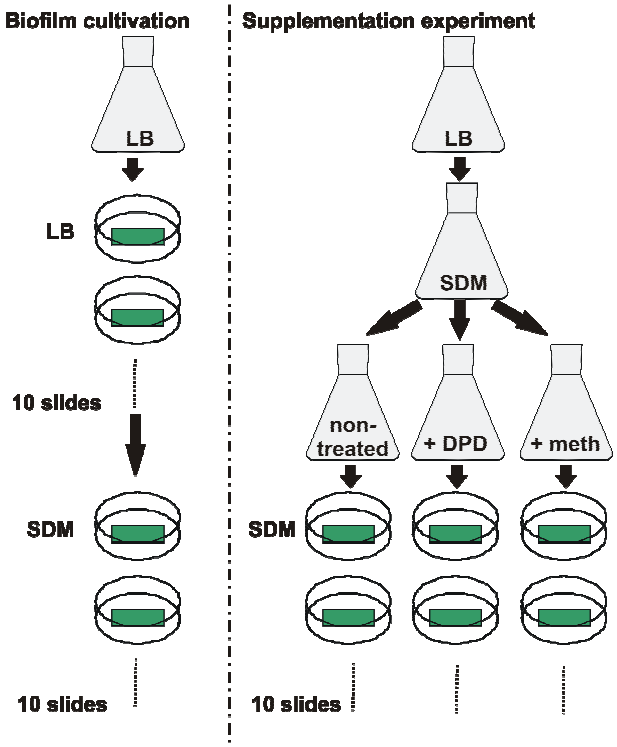


Figure 1-9. Experimental design of biofilm cultivation.
In the first experiment, the cultures were poured onto sterile glass slides in Petri dishes. These dishes were then cultivated for 45 min, and then the slides were transferred into fresh defined minimal medium (SDM) in Petri dishes (10 slides). In the supplementation experiment, the fresh SDM medium was inoculated from the LB cultures. The inoculated SDM medium was divided into three, and supplemented with DPD, methionine, or left unsupplemented. These latter solutions were transferred onto sterile glass slides (10 of each treatment).

3.10.1 Biofilm formation assay

(Stepanovic *et al.*, 2004)

The *luxS*$^{-}_{ins}$ mutant and WT_{Km} control strain were cultivated in LB medium for four hour to an OD_{600nm}=0.7-1. 1.5 ml of each culture was pipetted in six replicas into a 24-well microtitre plate. The control was sterile LB medium. After 45 minutes incubation, LB cultures and LB media were replaced by 1.5 ml fresh SDM medium. At each time point, one microtitre plate was removed for analysis.

For biomass measurement, the medium was first removed from the well. Then, 0.5 ml 1 % crystal violet dissolved in ethanol (p.a.) was added into each well and incubated at RT for 15 minutes. Then, the dye was removed, and the wells were washed with 1.5 ml deionised water. The washed wells were filled with 1.5 ml ethanol (p.a.). The microtitre plates were sealed with parafilm, fastened to a thermomixer and were slowly shaken overnight in order to extract the dye. The next day, OD_{620nm} was measured.

3.11 Secretome analysis

Sh. oneidensis luxS$^{-}_{ins}$ and WT_{Km} strains were cultivated at 30 °C in LB till OD_{600nm}=1.5 for 5-5.5 h. Then, the whole 100 ml culture was centrifuged at 4.000 x g for 6 minutes at 4 °C and the culture supernatant was sterilized by filtration (0,22 µm).

The precipitation and analysis of the proteins in the supernatant was performed in the group of Lothar Jänsch, Cellular Proteomic group/Helmholtz Centre for Infection Research. Proteins were precipitated by sodium deoxylate (DOC). Peptides were generated by trypsine digestion. The peptides were labelled by different iTRAQ reagents (Applied Biosystems) according to the manufacturer's instructions.

iTraq labelled peptides were first separated using strong cation exchange on a Mono S PC 1.5/5 column using an gradient over 35 minutes from 100 buffer A to 35% buffer B (A = 25% acetonitrile, 0.1% formic acid; B = 25 % acetonitrile, 0.1% formic acid, 500 mM KCl) at a flow rate of 150 µl/min. Fractions of 150 µl were collected, dried in a SpeedVac centrifuge and purified using ZipTipµ RP 18 material.

The separation of the peptide samples was performed using a bioinert Ultimate nano-HPLC system (Dionex). 10 µl of each sample (containing up to 1 µg peptides) were injected, and peptides were purified and concentrated on a C_{18}-PepMap pre-column (0.3 mm i.d. x 5 mm, 100 Å pore size, 3 µm particle size) at a flow rate of 30 µl/min in 0.1% TFA. Subsequently, peptides were separated on an analytical 75 µm i.d. x 150 mm C_{18}-PepMap column (Dionex, 100 Å pore size, 3 µm particle size) at a flow rate of 200 nl/min. The gradient (Solution A: 0.1% formic acid, 5% acetonitrile; solution B: 0.1% formic acid, 80% acetonitrile) started at 5% and ended at 60% B after 120 minutes.

MS and MS/MS data were acquired using a Q-TOFmicro mass spectrometer (Waters, Milford Massachusetts; USA). Doubly and triply charged peptide ions were automatically selected by the MassLynx software (MassLynxx 4.1 1b) and fragmented for a maximum of 18 seconds per peptide. MS data were automatically processed and peak lists for subsequent protein identification by database searches were generated by the MassLynx software. Database searches were carried out with an in house MASCOT server using the EMBL *Shewanella oneidensis* database. Proteins were only accepted as identified when at least one unique peptide showed an individual score above 20, which indicated identity or extensive homology ($p<0.05$) using the given settings (Enzyme: Trypsin; Max. missed cleavages: 1; Fixed modification: iTRAQ (K); iTRAQ (N-term); Oxidation (M); Peptide tolerance: 0.4 Da; MS/MS tolerance 0.3 Da).

3.12 Siderophore analysis

(Schwyn & Neilands, 1987)

This method functions with a test solution that contains a ternary complex from chrome-azurol S/ iron(III)/ hexadecyltrimethylammonium bromide. The complex exhibits a blue colour, which turns into orange upon iron removal by a strong chelator, like siderophores. The test solution can be applied for quantitating measurements in supernatants and for qualitative analysis in solid media. In this study, the test solution was supplemented to solid SDM medium to 1:10 ratio resulting in a deep green agar. This green colour instead of blue did not disturb the analysis. It is likely to have been caused by the trace metal content of the minimal medium on the basis of observations.

The SDM basic agar was prepared as regular but it was filled up to 870 ml. After autoclaving, the SDM basic medium was supplemented with the solutions for full SDM medium and the siderophore test solutions, as 40 ml HDTMA and 60 ml CAS/iron solution.

HDTMA (Hexadecyltrimethylammonium):
72.9 mg HDTMA was dissolved in 40 ml MilliQ water using one drop of 3 % HCl. The solution was autoclaved at 121 °C for 15 min.

CAS/iron:
93 mg chromeazurol S (CAS) was dissolved in some MilliQ water, and then 10 ml iron solution was added and filled up to 60 ml final volume. The bottle was wrapped in alufoil. The solution was autoclaved at 121 °C for 15 min.

Iron solution:
1mM $FeCl_3$-6-hydrat was dissolved in some MilliQ water, and then 83 µl 37 % HCl (10 mM final concentration) was added, and filled up to 100 ml final volume. The bottle was wrapped in alufoil.

For the siderophore test, overnight pre-cultures of *Sh. oneidensis luxS*$^{-}_{ins}$ and WT_{Km} strains were dropped (50 µl) on the surface of the green agar three times. Each day the plates were looked at for fading zones.

4 Results

4.1 Response of *P. aeruginosa* virulence genes to autoinducer-2

The culture supernatant of *Streptococcus* species CF004 induced 12 virulence genes of a clinical isolate *P. aeruginosa*, while synthesized AI-2 induced six out of 12 of these genes (Duan *et al.*, 2003). These six genes are listed in Table 4-1 with fold induction values of the *Streptococcus* species CF004 and AI-2 respectively. The fold induction was detected using the low-copy plasmids, pMS402, in which the promoter regions of the genes were fused to a luminescence reporter cassette.

Table 4-1. Induction of *P. aeruginosa* genes by *Streptococcus* species CF004 and by AI-2.

Gene	PA no.	Function/ description	Fold induction	
			CF004	AI-2
phzA2	PA1899	Phenazine synthesis cluster 2, first gene	7.1	4.3
lasB	PA3724	Elastase	6.0	2.4
phzA1	PA4210	Phenazine synthesis cluster 1, first gene	3.0	3.2
rhlA	PA3479	Rhamnolipid (rhamnosyltransferase chain A)	2.0	2.2
exoT	PA0044	Exoenzyme T (99% similar to ADP-ribosyltransferase)	1.9	1.9
fliC	PA1092	Flagellin type B	1.8	3.6

Two of these genes were tested: *phzA1* and *lasB*. Both genes are important virulence genes triggered by quorum sensing via the acylated homoserine lactone (AHL) signals of *P. aeruginosa*. The *phzA1* gene encodes pyocyanin, which is a blue-green pigment functioning as an electron shuttle and an antibiotic. The *lasB* gene encodes elastase, an extracellular enzyme, which degrades the human tissues thereby causing inflammation and suppresses the immune system.

To test if AI-2 induces these genes, AI-2 containing solutions were added to *P. aeruginosa* strains transformed with reporter plasmids under the control of promoters of the genes.

The first reporter plasmid was pMS402-*phzA* (kindly sent by Michael G. Surette) containing the *phzA1* promoter fused to a luminescence reporter gene. This plasmid was also used in the original study (Duan *et al.*, 2003) (Figure 4-1). The other reporter plasmid was pKR-C12 containing the *lasB* gene fused to a GFP reporter (Riedel *et al.*, 2001) (Figure 4-1). This plasmid has successfully been applied in discovering new AHLs with long acyl side chains (Wagner-Döbler *et al.*, 2005).

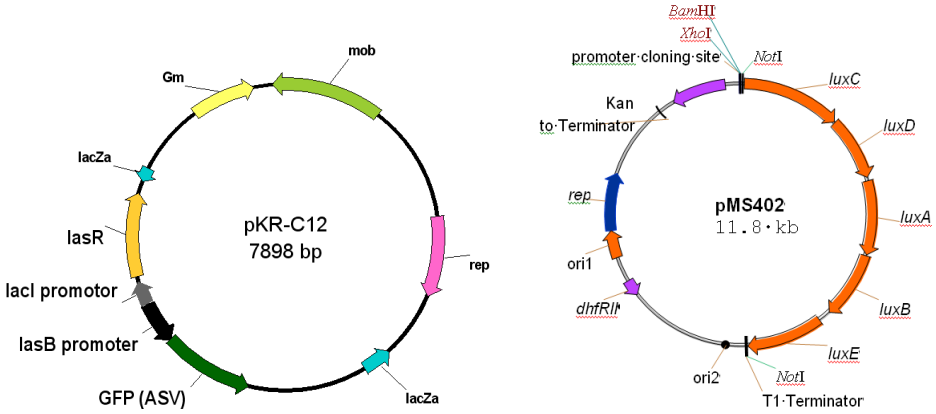


Figure 4-1. The pKR-C12 and the pMS402, low-copy plasmids.
The pKR-C12 plasmid contains an unstable GFP variant (GFP(ASV)) under the control of the *lasB* promoter. It also contains the *lasR* protein under the control of the *lacI* promoter, nevertheless this part was not used, since the plasmid was transformed into *P. aeruginosa* strains expressing LasR protein from the chromosome. Other components are the gentamicin resistance cassette (Gm), mobilization protein (mob) and replication protein (rep). Also the remnants of the disrupted *lacZa* protein were found. The sequence of the plasmid was obtained from Dr. Katrin Riedel.
The pMS402 plasmid contains the promoters of *P. aeruginosa* at the promoter cloning site fused to the *luxCDABE* genes responsible for luminescence in response to quorum sensing signals. In addition, the plasmid encodes for kanamycin resistance (Kan). The figure of the plasmid was found on a poster published on the internet.

Into the $PAO1_{Igl}$ wildtype strain, the pMS402- *phzA1* and the pKR-C12 plasmid were introduced separately. Into the PAO1-$MW1_{lasI^-, rhlI^-}$ strain, the pKR-C12 plasmid was introduced. In the case of the other two strains, $PAO1_{O/V}$ and PAO1 $_{lasR^-, rhlR^-}$, plasmid transformation was not completed. These strains came from the same laboratory and could have been transformed exclusively by electroporation.

Thereafter, AI-2 containing solutions were assayed for promoter induction in the reporter plasmids in the *P. aeruginosa* strain.

In the $PAO1_{Igl}$ wildtype strain, AI-2 containing solutions induced neither the *lasB* nor the *phzA1* promoters (data not shown). Even the control autoinducer, C12-HSL (N-3-oxo-dodecanoyl-L-homoserine lactone), induced these promoters very slightly with high

standard deviations. Conceivably, the autoinducer produced by the wildtype strain affected the bioassays.

In the PAO1-MW1$_{lasI^-, rhlI^-}$ strain, the bioassays were undertaken with two different kind of AI-2 containing solution (Figure 4-2 and Figure 4-3).

As long as synthetic AI-2 (also named DPD) was not available, the culture supernatant of *V. harveyi* BB152 was used as an AI-2 containing solution. The negative control was the culture supernatants of *V. harveyi* MM77, a mutant which is unable to produce AI-2.

C12-HSL in LB induced the *lasB* promoter about 5-fold, whereas the culture supernatants of both *V. harveyi* mutants did not exhibit any induction (Figure 4-2). Although C12-HSL induced the promoter somewhat more in combination with the culture supernatant of *V. harveyi* or AB medium, this effect was attributed to the AB medium. Namely, AB is a nutrient-arm medium compared to LB and it could cause less inhibition on the expression of quorum sensing genes.

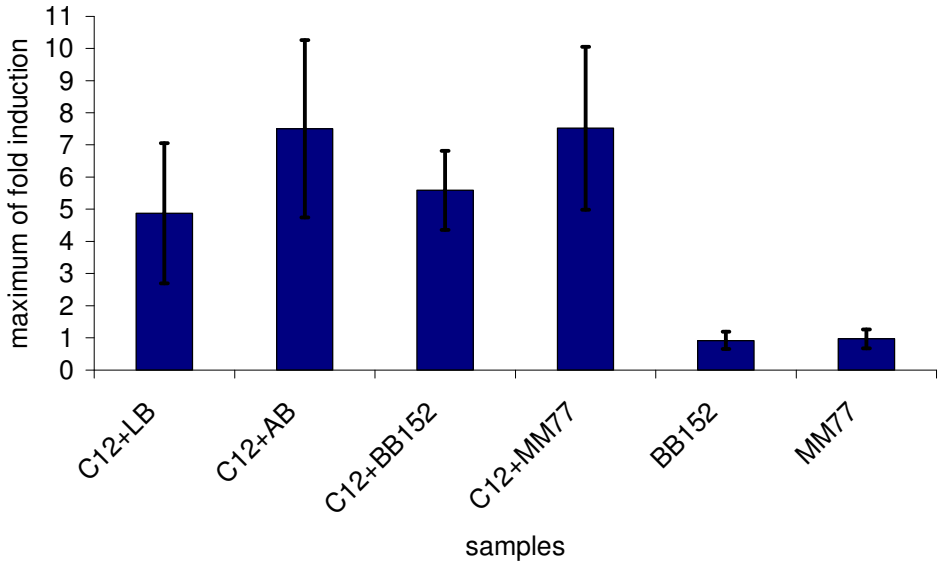


Figure 4-2 Response of the *lasB* promotor in *P. aeruginosa* PAO1-MW1$_{lasI^-, rhlI^-}$ to culture supernatants of *V. harveyi*.
C12-HSL in LB, the positive control, induced the test strain about 5 fold. C12-HSL in AB medium or in the *V. harveyi* BB152 and MM77 supernatants prepared in AB caused fold induction above the positive control. The *V. harveyi* BB152 containing AI-2 and MM77 lacking AI-2 supernatants alone caused no induction in the test strain.

Later, as synthetic DPD was available, this bioassay was repeated. Two concentrations of DPD were tested: 5.3 µM, routinely used in the *V. harveyi* bioassay and representing the regular autoinducer concentration, and 53 µM, 10 fold concentration.

C12-HSL induced the promoter about 10 fold, whereas DPD at both concentrations did not induce the *lasB* promoter. C12-HSL combined with DPD at either concentration did not exhibit higher induction (Figure 4-3).

Therefore in the PAO1-MW1$_{lasI^-, rhlI^-}$ strain, AI-2 did not induce the *lasB* promoter.

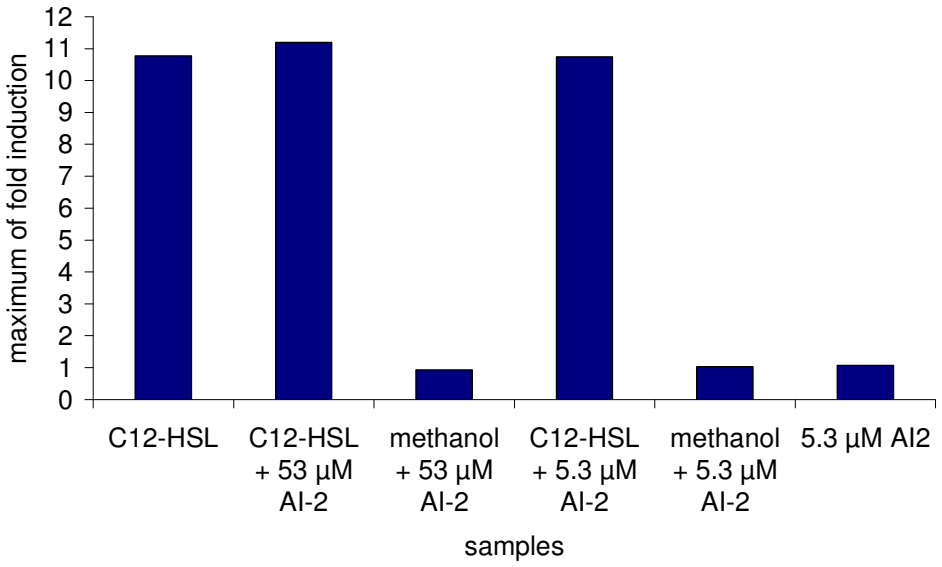


Figure 4-3. Response of the *lasB* promoter in *P. aeruginosa* PAO1-MW1$_{lasI^-, rhlI^-}$ to synthetic DPD.
Alone or combined with DPD at either concentration, C12-HSL, Quorum sensing signal induced the *lasB* promoter at a similar level. Alone, DPD at both concentrations did not induce the *lasB* promoter.

In conclusion, the AI-2 containing solutions induced neither *lasB* nor *phzA1* promoters in *P. aeruginosa* PAO strain, and thus the previous study could not be reproduced.

4.2 Phylogenetic distribution of the Pfs/LuxS and SahH pathways

The phylogenetic distribution of the Pfs/LuxS pathway and the alternative SahH pathway were investigated in non-pathogenic, marine bacteria (Allgaier *et al.*, 2003; Labrenz *et al.*, 1999; Uphoff *et al.*, 2001). To prove the presence of Pfs/LuxS pathway and SahH pathways, the *luxS* and *sahH* genes were identified using PCR with degenerated primers respectively.

Habitat. The marine bacteria were isolated from different habitats of the North Sea near the island of Helgoland, namely from the surface of microalgae (diatoms, dinoflagellates) and macroalgae (laminaria), suspended particles, *in situ* grown biofilms and the picoplancton. In addition, eight strains from the hypersaline Antarctic Ekho Lake were included.

Phylogeny. The 164 isolates were identified on the basis of near complete 16S rRNA gene sequences (Appendix A) and belonged to three different phylogenetic groups: *Alphaproteobacteria* (71 isolates), *Bacteroidetes* (64 isolates) and Gammaproteobacteria (64 isolates). Each phylogenetic group was represented in each niche, except that the bacteria from the Ekho Lake were all *Alphaproteobacteria*. In order to confirm the result on marine *Shewanella* species, eight type strains of the genus *Shewanella* and of *Alishewanella fetalis* were investigated additionally.

PCR. Two degenerated primer pairs amplified the *luxS* and *sahH* genes respectively. The first primer pairs were phylogenetically independent and they were LuxS_degfor3 and LuxS_degrev4 for the *luxS* gene and SahH_degfor and SahH_degrev for the *sahH* gene. If these general primers did not work, other phylogenetically specific primer pairs were used. These were: LuxS_gammafor1 and LuxS_gammarev2 for the *luxS* gene in *Gammaproteobacteria* and SahH_alphafor and SahH_alpharev for the *sahH* gene in *Alphaproteobacteria* (Primer design is described in Materials and Methods.)

The amplicons of the isolates were compared to that of the positive control, and in cases where they were of the correct length, they were identified as potential *luxS* or *sahH* genes. The positive control for the *luxS* gene was the DNA of *V. harveyi* BB152, while the positive control for the *sahH* gene was the DNA of *P. aeruginosa* for *Gammaproteobacteria* and the DNA of *Rhodobacter litoralis* for other bacteria. Several amplicons, at least one per genus, were confirmed by sequencing (Appendix A).

Figure 4-4 summarizes the result of both genes in marine and *Shewanella* species.

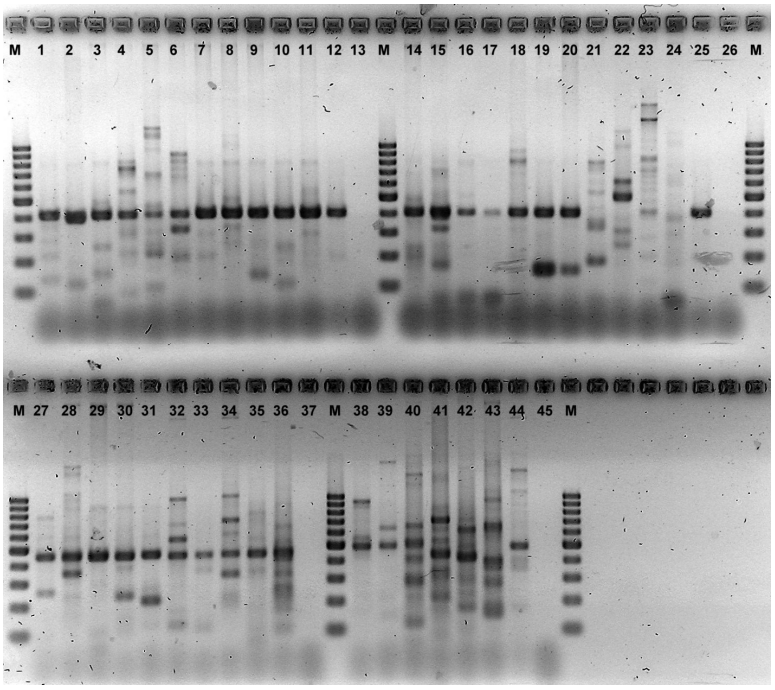


Figure 4-4. Identification of *luxS* and *sahH* genes in marine and *Shewanella* species.
The first and second row displays the result of the screening for *luxS* and *sahH* respectively.
Lane 1-11: *luxS* gene in *Alishewanella* and *Shewanella* type strains; 14-20: *luxS* gene in *Gammaproteobacteria*; 21-24: *Gammaproteobacteria* without the *luxS* gene; 27-35: *sahH* gene in *Bacteroidetes* and *Alphaproteobacteria*; 38-39: *sahH* gene *in Gammaproteobacteria*; 40-43: *Gammaproteobacteria* without the *sahH* gene. Lanes 12, 25, 36 and 44 represent the positive controls respectively. Lane 13, 26, 37 and 45 represent the negative controls respectively.
1. *Alishewanella fetalis*; 2. *Shewanella algae*; 3. *Sh. fidelis*; 4. *Sh. frigidimarina*; 5. *Sh. japonica*; 6. *Sh. livingstonensis*; 7. *Sh. marinintestina*; 8. *Sh. oneidensis*; 9. *Sh. sairae*; 10. *Sh. schlegeliana*; 11. *Sh.violaceae;* 12. *V. harveyi* BB152; 14. *Sh.oneidensis* DT-1; 15. *Sh.baltica* DT-11; 16. *Shewanella* sp. BIO-50; 17. *Halomonas* sp. HEL-68; 18. *Pseudoalteromonas* sp. HEL-40; 19. *Vibrio sp.* BIO-249; 20. *Vibrio sp.* BIO-305; 21. *Pseudoalteromonas sp.* DT-9; 22. *Halomonas* sp. PIC-155; 23. *Marinobacter sp.* DFL-32; 24. *Glaciecola* sp. BIO-261; 25. *V. harveyi* BB152; 27. *Aquimarina* sp. LM-17; 28. *Donghaena* sp. DT-2; 29. *Krokinobacter genikus* LM-2; 30. *Rhodovirga* sp. DFL-18; 31. *Venteria marina* PIC-1; 32. *Dinoroseobacter shibae* DFL-12; 33. *Jannaschia helgolandensis* HEL-10; 34. *Roseovarius mucosus* DFL-35; 35. *Sphingomonas baekryungensis* HEL-9; 36. *Rhodobacter litoralis*; 38. *Psychrobacter galcincola* HEL-6; 39. *Psychrobacter immobilis* HEL-44. 40. *Pseudoalteromonas sp.* DT-9; 41. *Halomonas* sp. PIC-155; 42. *Marinobacter sp.* DFL-32; 43. *Glaciecola* sp. BIO-261; 44. *Pseudomonas aeruginosa*

4.2.1 Alphaproteobacteria and *Bacteroidetes*

In 66 out of 71 *Alphaproteobacteria* (93 %), the *sahH* gene was identified. In five *Alphaproteobacteria*, neither of the genes could not be amplified. This latter, inconclusive PCR result could have been caused by primer mismatch, since the *sahH* gene was found in closely related isolates, and within this clade, the lack of both genes has only been seen in the parasitic *Ricksettia* species, and none of these tested species were parasitic. In all 29 *Bacteroidetes* strains, the *sahH* gene was identified consistent with nine out of 11 complete *Bacteroidetes* genomes.

In conclusion, the *Alphaproteobacteria* and *Bacteroidete*s isolates had the *sahH* gene and they do not have the potential to communicate via AI-2.

4.2.2 Gammaproteobacteria

In the 64 marine *Gammaproteobacteria*, some genera contained the *luxS* gene, while others the *sahH* gene or neither of the genes.

***luxS* gene.** The *luxS* gene was found in all strains of *Alteromonas*, in the marine *Shewanella* (three strains) and *Vibrio* (three strains) isolates. Complete genome sequences are available in the latter two genera, and this result was consistent with them. In order to determine if the *luxS* gene was consistently present in the *Shewanellaceae* family, nine type-strains and *A. fetalis* were investigated. In all of them the *luxS* gene could be amplified and verified by sequencing.

Furthermore, the *luxS* gene was found in seven out of 13 strains of *Halomonas* species. No complete genome sequence is available in this genus yet. An amplicon of identical length to the *luxS* gene was found in 12 out of 28 *Pseudoalteromonas* isolates, and one of them was confirmed by sequencing to be the *luxS* gene fragment. The presence of the *luxS* gene in the *Pseudoalteromonas* species is new, since the two complete genome sequences of *P. haloplanktis* and *P. atlantica* contain only the *pfs* gene.

***sahH* gene.** The *sahH* gene was found in three strains of the *Psychrobacter* species, consistent with the two complete genomes. The *sahH* gene is also present in the genome of *Idiomarina loihiensis* and *Saccharophagus degradans* that represent other marine genera from *Gammaproteobacteria*.

Lack of both genes. 27 of the 64 *Gammaproteobacteria* had neither the *luxS* nor the *sahH* gene. Ten of them belonged to the genera *Glaciecola*, *Halomonas* or *Marinobacter*. Genome sequences from *Glaciecola* and *Halomonas* species are not yet available; nevertheless the genome sequence of *Marinobacter aquaeolei* contained the *sahH* gene. Seventeen strains having neither of the genes belonged to the genus *Pseudoalteromonas*, consistent with the two complete genomes.

The identity of the *luxS* fragment was occasionally difficult to confirm, because fragments of a similar size had been simultaneously amplified. Therefore, it cannot be excluded that more *luxS* and *sahH* genes might have been determined using different primers, more specific to these genera.

In conclusion, the presence or the absence of the *luxS* and *sahH* genes was consistent within one genus and with the completely sequenced species. Inconsistency was found in the genera *Halomonas*, *Pseudoalteromonas* and *Marinobacter*.

4.3 AI-2 production of *Shewanella* species

AI-2 production was investigated in eight *Shewanella* type-strains, in the type-strain of *Alishewanella fetalis* and in one isolate from our laboratory, *Shewanella hafniensis* DT-1. All species contained the *luxS* gene in accordance with the available genome sequences and as confirmed by PCR in the previous section.

Sh. algae was isolated from red alga as well as from clinical specimens. It is a mesophilic, opportunistic human pathogen, whose natural environment is the sea. *Sh. frigidimarina* was isolated from the Antarctic sea and it is a phychrotrophic marine species. *Sh. fidelis* was isolated from sea sediments and it is a mesophilic marine species possessing haemolytic activity. *Sh. japonica* was isolated from sea water and mussels and it is a mesophilic species possessing haemolytic and cytotoxic activities. *Sh. marinintestina*, *Sh. schlegeliana*, and *Sh. sairae* were isolated from the intestines of fishes and other sea animals and they are phychrophillic species. *Sh. oneidensis* was isolated from the sediment of a fresh water lake as well as from clinical specimens and it is a mesophilic fresh water species. *A. fetalis* was isolated from a human foetus and it is a mesophilic species very similar to *Shewanella*. *Shewanella* sp. DT-1 was isolated from the surface of the diatom Thalassiosira sp. that was picked from a plankton sample (170 µm net) collected near the island of Helgoland in the North Sea on 4th April 2002 (depth 0.5 – 1.5 m).

Each strain showed AI-2 activity thereby confirming that *Shewanella* species do produce AI-2 in accordance with on the presence of *luxS*. The strains produced AI-2 at different maximum levels but with similar patterns. Although the species were isolated from different habitats, there was no correlation between habitat and AI-2 level.

4.3.1 AI-2 level

The AI-2 activity was detected using the *V. harveyi* bioassay in the culture supernatants of the species, which were collected during growth. The maximum level of AI-2 activity (AI-2 level) appeared at the end of the logarithmic phase. Table 4-2 shows the summary of all 73 bioassays conducted; for each species, the AI-2 level is averaged for the indicated number of bioassays.

Table 4-2. Mean maximum of AI-2 activity in *Shewanella* type strains and isolate DT-1.
The peak of AI-2 activity (in % of positive controls) during one complete growth curve is averaged for the indicated number of bioassays. Mean ± standard deviation are given.

Strain	Species	Peak A1-2 activity [% pos. contr.]	no of bioassays
CCUG 30811^T	*Alishewanella fetalis*	17.7 ± 30.4	5
DSM 9167^T	*Shewanella algae*	8.2 ± 10.2	9
LMG 20552^T	*Shewanella fidelis*	18 ± 6	8
LMG 18921^T	*Shewanella frigidimarina*	7.5 ± 6	6
LMG 19691^T	*Shewanella japonica*	46.2 ± 33	4
LMG 21403^T	*Shewanella marinintestina*	3.8 ± 3	10
ATCC 700500^T	*Shewanella oneidensis* MR-1	9.8 ± 9	10
LMG 21408^T	*Shewanella sairae*	34 ± 38	5
LMG 21406^T	*Shewanella schlegeliana*	5.8 ± 5	5
DT-1[2)]	*Shewanella hafniensis*	4.3 ± 1	4

The AI-2 levels were between 4% (e.g. *Shewanella hafniensis* DT-1, *Sh. marinintestina*) and 46% (*Sh. japonica*) of the positive controls (synthetic DPD or culture supernatant from *V. harveyi* BB152). The level was under 10 % in six species, 18 % in two species, and 34 % and 46 % in two species respectively. Figure 4-6 shows a representative subset of these data. For each species, two different cultures are shown, each of which was tested in two different bioassays independently.

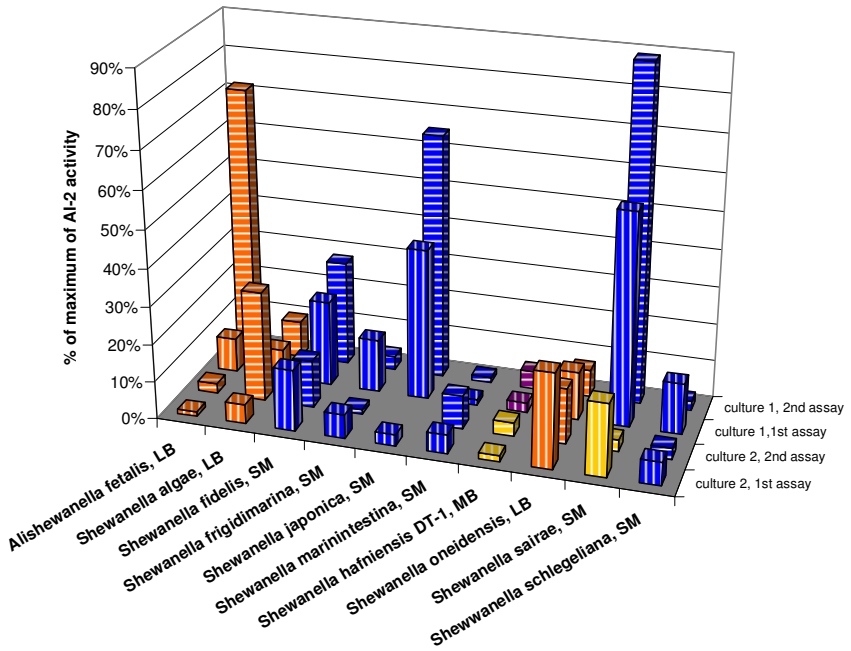


Figure 4-5. AI-2 production in different species of *Shewanella*.
Maximum level of AI-2 production during growth in different species of *Shewanella* and *Alishewanella* determined by the *V. harveyi* bioassay. AI-2 production is indicated as percent of positive controls (synthetic DPD or culture supernatant from *V. harveyi* BB152). Culture supernatants of the strains were collected from two different cultures throughout growth (culture 1 and culture 2), and tested in the bioassay twice (1st assay and 2nd assay). The colour of the bars indicates the type of cultivation medium used: blue: SM medium; orange: LB medium; yellow: MB medium; violet: LBSS medium.

Inhibition of LB and SM media

Most of the culture supernatants were prepared in LB and SM medium. Both are rich in nutrients, which can inhibit the luminescence of the *V. harveyi* sensor strain, and thus decreasing the AI-2 activities. According to our earlier experience, the more nutrients a culture supernatant contained, the more the luminescence of the sensor strain was inhibited.

The LB medium substantially inhibited the luminescence of the *V. harveyi* sensor strain, while the SM medium did not. This luminescence inhibition was measured in the following way. AB, LB and SM media and culture supernatants were supplemented with DPD at 5.3 µM final concentration respectively. AB medium was the ideal medium for the *V. harveyi* bioassays and 5.3 µM DPD in AB commonly caused maximal AI-2 activity. Compared to DPD in AB, DPD in LB had only about 40 % AI-2 activity, while DPD in SM had approximately 100 % AI-2 activity (data not shown).

As a consequence, the species grown in LB, as *A. fetalis, Sh. algae* and *Sh. oneidensis*, must produce more AI-2 than shown above, but because of the nutrient content of their culture supernatants less AI-2 activity is detected in the *V. harveyi* bioassay.

High variability

Although the AI-2 activity of each culture supernatant was normalized with the AI-2 activity of the positive controls, the variability of AI-2 activity between cultures and between replicate bioassays was sometimes very high. The reason is that the bioassay result is dependent on the growth of two living bacteria, the Shewanella sp. which produced AI-2 into the culture supernatants and the *V. harveyi* which detected AI-2.

4.3.2 AI-2 pattern

Independent of the level of AI-2 activity detected, the same growth phase dependent pattern of AI-2 production (AI-2 pattern) was observed in all investigated strains.

In all *Shewanella* strains, AI-2 activity reached its maximum towards the end of the exponential growth phase and decreased during the stationary phase (Figure 4-6). In species with high AI-2 activity, this decrease occurred gradually. In contrast, if the AI-2 activity was low, it disappeared rapidly at the beginning of the stationary phase within 2–4 hours. At the late stationary phase (after 24 h of growth), no AI-2 activity was detected in any of the strains tested.

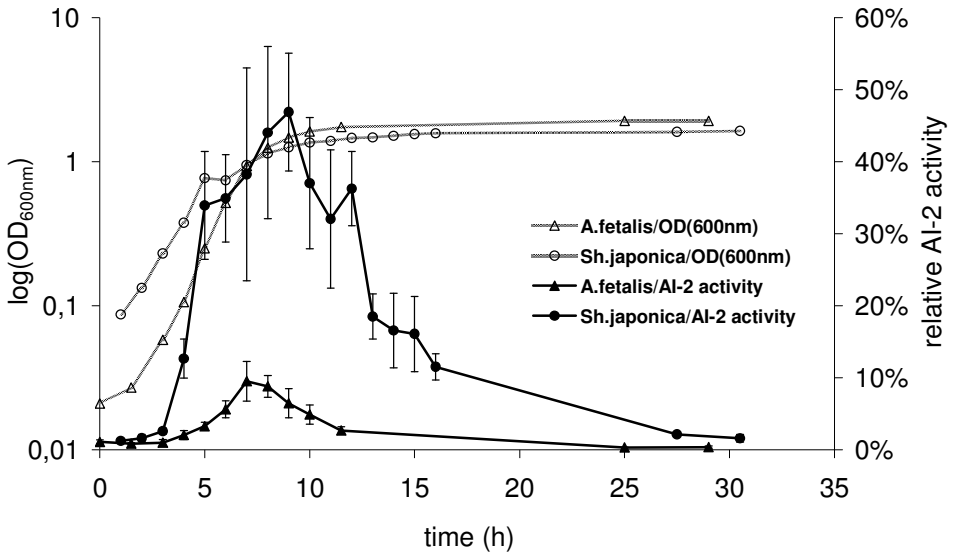


Figure 4-6. Pattern of AI-2 activity during growth in *Alishewanella fetalis* and *Sh. japonica*.
Species, like *Sh. japonica*, which produced high AI-2 activity, gradually decreased AI-2 activity in the stationary phase, in contrast to species, like *A. fetalis*, which produced low AI-2 activity, decreased AI-2 activity within a couple of hours. In both species, AI-2 activity accumulated in the exponential phase, and peaked at the end of the logarithmic phase.

4.3.3 AI-2 production in different media

The same level and pattern of AI-2 activity were observed in all cultivation media used under aerobic conditions.

A. fetalis, *Sh. algae* and *Sh. oneidensis* were cultivated also in SM medium in addition to LB. *A. fetalis* and *Sh. algae* reached lower cell-densities in this marine medium and had 34.7 ± 7 % and 11.8±9.7 % AI-2 activity respectively. These AI-2 activities were lower than observed in LB medium, but they were in accordance with poorer growth. *Sh. oneidensis* did not exhibit growth in this medium probably due to high salt concentration.

Sh. hafniensis DT-1, since it is one of the less-known isolates, was cultivated also in two different marine media, in LBSS and in MB. In both media, it grew similarly and had similar AI-2 activities (Figure 4-7).

Sh. oneidensis was also cultivated anaerobically using iron as an electron acceptor (with 10 and 20 mM ferric citrate, respectively), but under these conditions, its growth was very slow and AI-2 activity was not found (data not shown).

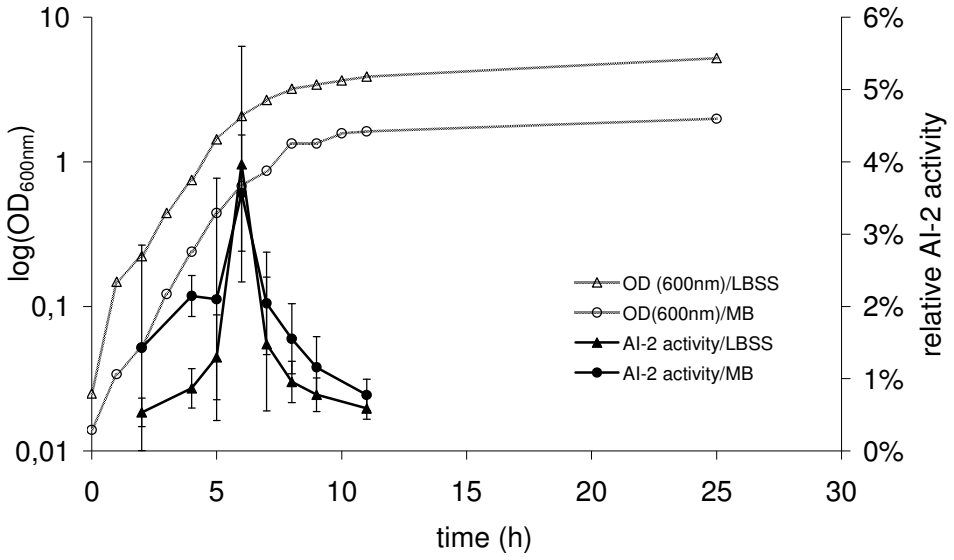


Figure 4-7. AI-2 production of *Shewanella hafniensis* DT-1 in two different media.
Sh. hafniensis DT-1 was grown in LBSS and MB. In both media, it produced AI-2 activity at the same level and with the same pattern.

4.3.4 Comparison with *V. harveyi*

V. harveyi is the model organism for AI-2 signalling, and therefore its AI-2 production was compared with the AI-2 production of the *Shewanella* species in level and in pattern.

AI-2 level.

To facilitate the comparison of the level of AI-2 activities, the culture supernatants of *V. harveyi* was serially diluted and tested for AI-2 activity. Figure 4-8 shows the maximum level of AI-2 activity of each dilution.

The level of AI-2 activity appeared as a saturation curve in the function of the dilution grade, i.e. AI-2 amount, indicating a detection limit for the bioassay. 100 % of AI-2 activity was detected with culture supernatant undiluted to diluted 0.5-fold and thus *V. harveyi* produced double the amount of AI-2 as the detection limit. Linearly decreasing AI-2 activity was detected with culture supernatant diluted 0.1 to 0.5- fold (Figure 4-8).

The *Shewanella* species produced maximally 46 % of AI-2 activity. This corresponded to 0.2-0.3-fold diluted culture supernatant of *V. harveyi*, therefore the *Shewanella* species produced at least 4-5-fold less AI-2 than *V. harveyi*. Most *Shewanella* species produced less than 10 % of AI-2 activity corresponding to 0.1-fold diluted culture supernatant of *V. harveyi*, therefore these *Shewanella* species produced 10-fold less AI-2.

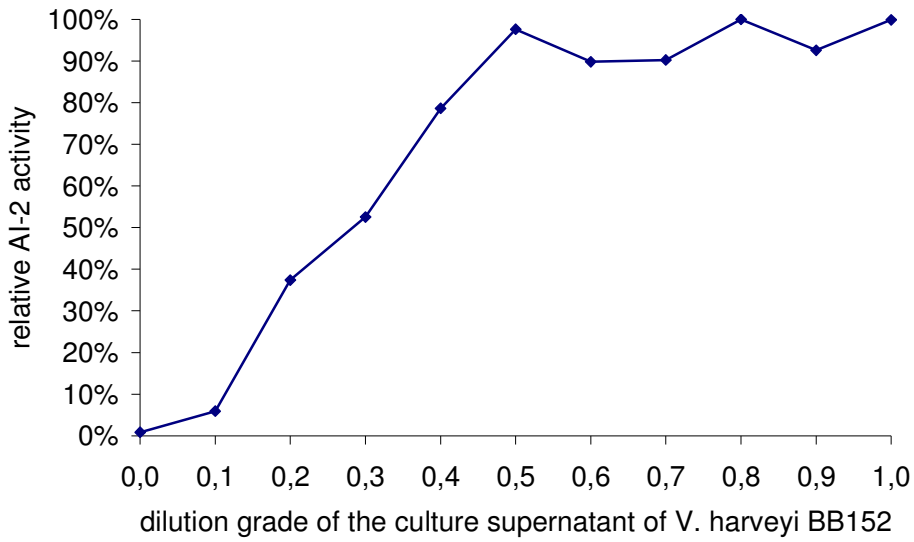


Figure 4-8. Relative AI-2 activity of serially diluted culture supernatant of *V. harveyi* BB152.
From 0.5 fold to undiluted culture supernatant of *V. harveyi* had 100 % of AI-2 activity. From 0.1 to 0.5 fold diluted culture supernatants had linearly decreasing AI-2 activity.

To estimate the concentration of produced AI-2, DPD at different concentrations was tested for AI-2 activity. In this case, the standard curve was somewhat different (Figure 4-9).

DPD at 5.3 mM had no AI-2 activity and thus it must inhibit at such a high concentration surplus. DPD from 530 µM to 5.3 µM had moderately decreasing AI-2 activity. DPD from 5.3 µM to 0.53 µM had abruptly decreasing AI-2 activity. Minor differences in concentration did not remarkably modify the AI-2 activities (data not shown).

Although the plateau observed with the culture supernatant of *V. harveyi* did not reappear, the contrast of the two curves gives the impression that 0.5 dilution of the culture supernatant of *V. harveyi* could correspond to DPD solutions at low µM range. Since the *Shewanella* species produced at least 4-5 fold less AI-2 than *V. harveyi*, they might produce AI-2 in low µM and in high nM range.

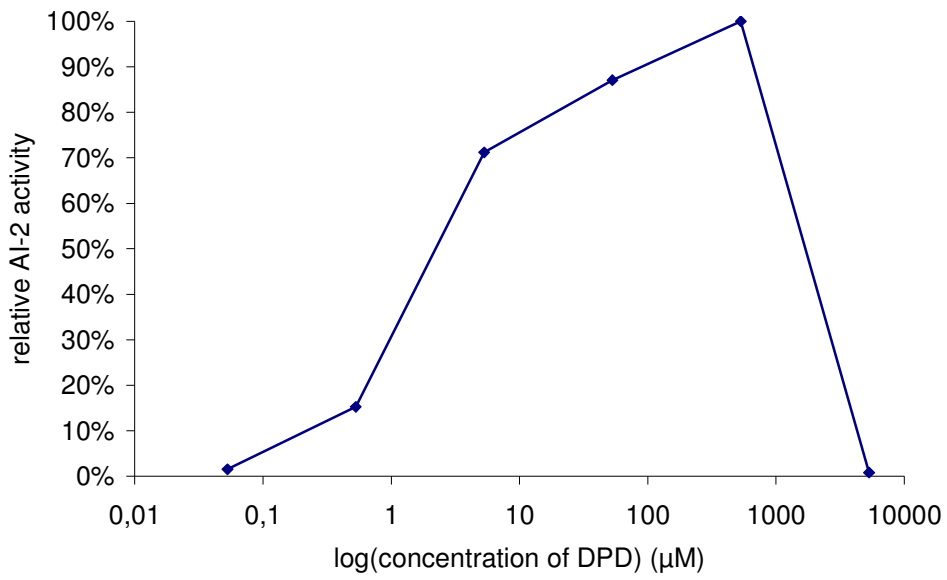


Figure 4-9. Maximum AI-2 activity of DPD at different concentrations.
DPD at 5.3 mM inhibited the luminescence of *V. harveyi* and no AI-2 activity was detected. DPD between 530 µM and 5.3 µM had moderately decreasing AI-2 activity. DPD between 5.3 µM and 0.53 µM had linearly decreasing AI-2 activity. DPD under 0.53 µM had less than 20 % of AI-2 activity.

AI-2 pattern.

The pattern of AI-2 activity of *V. harveyi* was different to that of the *Shewanella* species (Figure 4-10). The AI-2 activity of *V. harveyi* reached its maximum in the stationary phase and then remained steady, unlike that of the *Shewanella* species, which reached its maximum at the end of the logarithmic phase, and then decreased.

In conclusion, this observation supports the model that *V. harveyi* senses only AI-2. In AI-2 production and decrease, the *Shewanella* species resembles rather *S. typhimurium*, a species which takes AI-2 up at the end of the logarithmic phase.

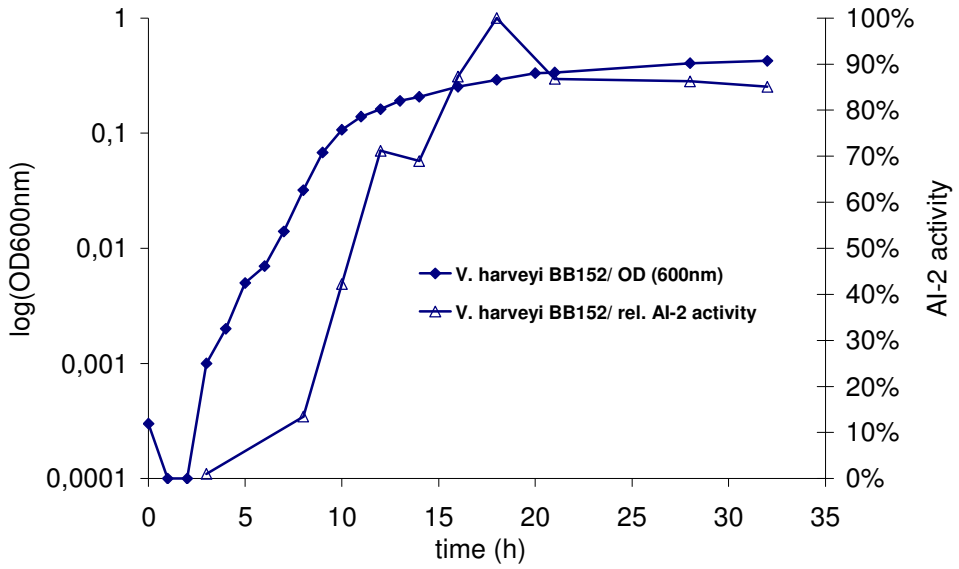


Figure 4-10. AI-2 production of *V. harveyi* BB152 during growth in AB medium.
AI-2 activity of *V. harveyi* reached its maximum in the stationary phase, and then remained steady, not like AI-2 activities of the *Shewanella* species, which reached their maximum at the end of the logarithmic phase and then decreased.

4.3.5 Inhibitory activity of *Sh. algae* and *Sh. oneidensis*

An interesting phenomenon was observed with stationary phase culture supernatants of *Sh. oneidensis* and *Sh. algae*. Namely, both *Shewanella* species inhibited the luminescence upturn of *V. harveyi* BB170 induced by its own AI-2 during the bioassay.

At the beginning of the bioassay (Figure 4-11), AI-2 of BB170 is at low concentration and externally added AI-2 induces its luminescence thus enabling measurement of the AI-2 activity of other species. In the later hours of the biossay, AI-2 of BB170 reaches the threshold level and induces its luminescence. This luminescence upturn was inhibited by *Sh. algae* and *Sh. oneidensis*.

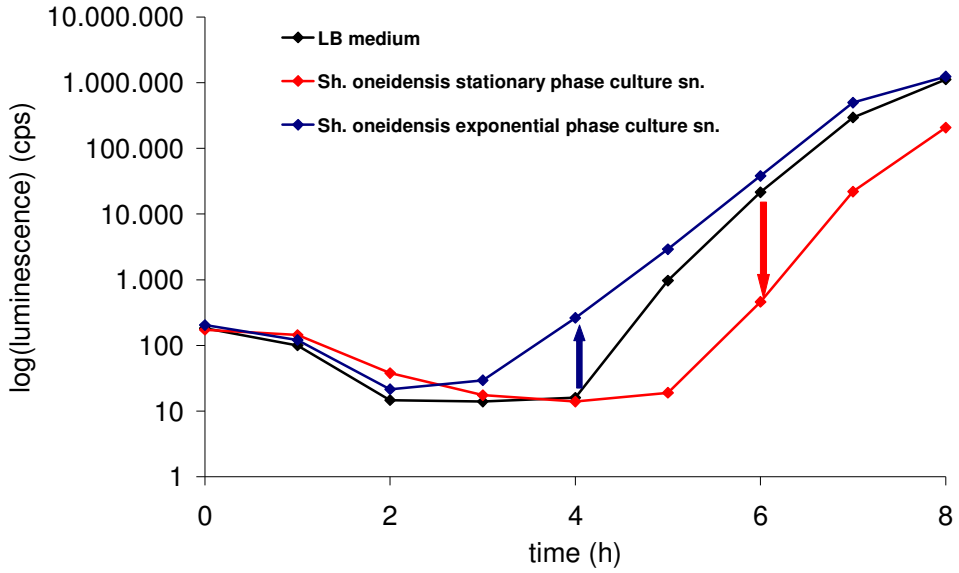


Figure 4-11. Induction and inhibition of *Sh. oneidensis* culture supernatants on the luminescence of *V. harveyi*.
When AI-2 of *V. harveyi* was at low concentration, the logarithmic phase culture supernatants induced the luminescence of *V. harveyi* (blue arrow), whose fold change gave the AI-2 activity. In contrast, when AI-2 of *V. harveyi* had accumulated and *V. harveyi* begin intensively luminescencing, the stationary-phase culture supernatants inhibited the luminescence of *V. harveyi* (red arrow).

To determine if the luminescence inhibition was caused by inhibited AI-2 signalling of *V. harveyi*, stationary phase culture supernatant of *Sh. oneidensis* was added to the culture of *V. harveyi* BB170 sensing only AI-2 as well as to the culture of *V. harveyi* BB886 sensing only AI-1, and then growth and luminescence of the cultures were monitored.

Luminescence inhibition was observed with both of the *V. harveyi* sensor strains, and therefore it was not caused by inhibited AI-2 signalling of *V. harveyi*. In addition, luminescence inhibition was observed only with *V. harveyi* cultures in the logarithmic phase, therefore it is a temporary effect. Since the growth of the cultures was also temporarily inhibited in the logarithmic phase, luminescence inhibition must be an indirect effect of growth delay.

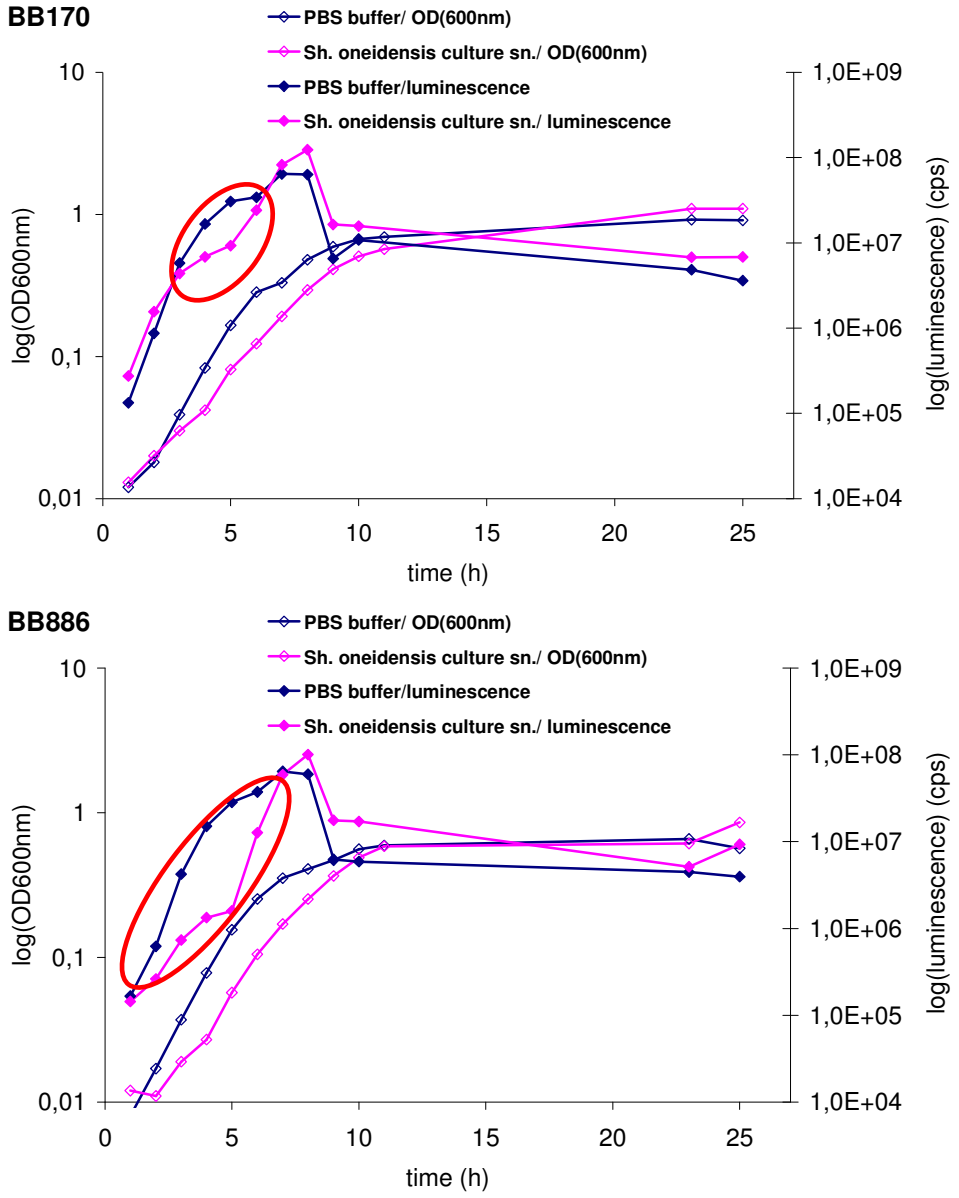


Figure 4-12. *Sh. oneidensis* culture supernatant temporarily inhibited luminescence and growth of the *V. harveyi* sensor strain.
BB170 senses only AI-2, BB886 senses only AI-1. Both were temporarily inhibited in growth and in luminescence by *Sh. oneidensis* stationary phase culture supernatant. Luminescence inhibition seems to be the consequence of growth inhibition. The red circles indicate the luminescence inhibition observed in the bioassay.

4.4 Construction and characterization of *Sh. oneidensis luxS* mutant

The *Sh. oneidensis luxS* knockout mutants were constructed by two methods. In the first method, an insertional mutant, $luxS^-_{ins}$, and an insertional control strain, WT_{Km}, were constructed. These strains were compared for phenotypic differences. In the second method, a deletion mutant without antibiotic marker was constructed, which was completed at the end of this work, and it has not been compared with the wildtype for phenotypic changes.

4.4.1 Construction of *Sh. oneidensis luxS* mutants

4.4.1.1 The *luxS* gene

The *luxS* gene of *Sh. oneidensis* is the SO.1101 gene located between 1.143.141 and 1.143.650 bp on the chromosome. It attains a length of 510 bp and can be translated into 169 amino acids. It contains also the consensus motif (d*spmGc*TG*y) in the middle of the amino acid sequence, as observed in the ClustalW alignments which might have an important function. The *luxS* gene is flanked by the extracellular solute binding protein (*esbp*) and a TonB dependent receptor (*tonB*).

4.4.1.2 Homologous recombination

Homologous recombination is a process, in which two DNA double-strands do crossover and exchange genetic material along their similar or identical DNA segments. In knockout mutant construction, a non-replicating, also named suicide, plasmid contains a fragment homologous to an appropriate segment of the genome. This plasmid is introduced into the cell and recombines with the genome under antibiotic pressure.

To construct a mutant by single homologous recombination (Figure 4-13), the suicide plasmid contains one fragment homologous to a region of the genome. During recombination, the homologous fragment is duplicated and, in the final construct, present at each end of the integrated plasmid. If the homologous fragment is an internal part of the target gene, the plasmid insertion interrupts it.

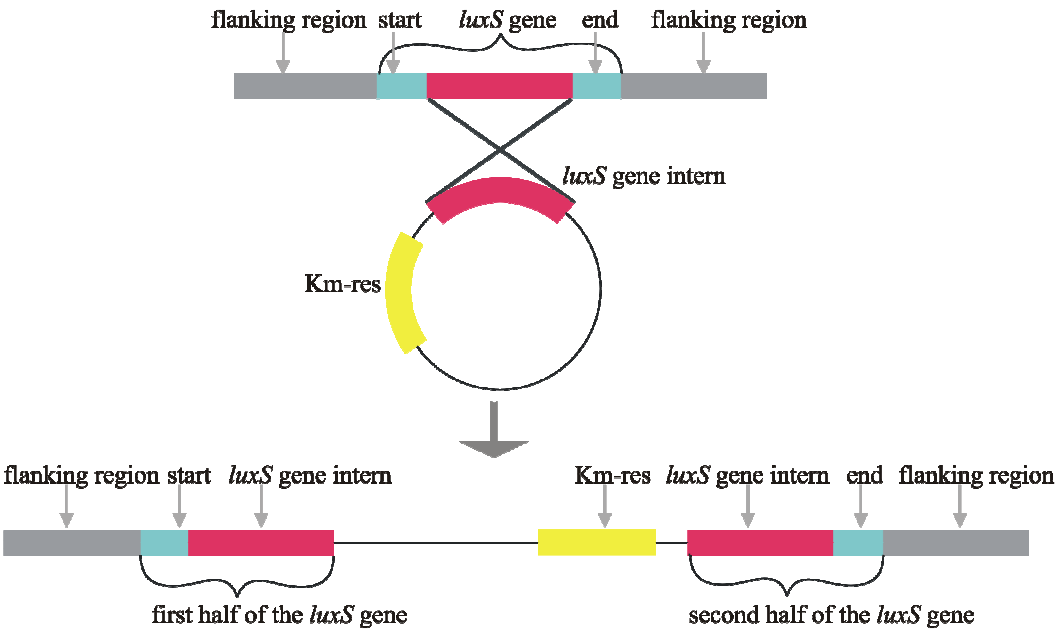


Figure 4-13. Scheme of constructing a single homologous recombination mutant.
The suicide plasmid contains an internal part of the target gene (red) and an antibiotic resistance cassette (Km-res: kanamycin, yellow). Due to kanamycin pressure, the cells integrate the plasmid into the genome via homologous crossover. In the final construct, the homologous internal part of the gene is duplicated and the target gene is present with a truncated first and second half on the genome. The plasmid backbone resides between the disrupted gene parts.

To construct a knockout mutant by double homologous recombination (Figure 4-14), the suicide plasmid contains fragments homologous to the flanking regions of the target gene, an antibiotic selection marker between the flanking regions (in this case gentamicin-green fluorescence cassette, Gm-GFP) and a counter selection marker.

The counter selection marker was the *sacB* gene. The *sacB* gene originates from *Bacillus subtilis* and encodes the levansucrase enzyme. If the *sacB* gene is expressed in a Gram negative bacterium which is unable to utilize sucrose, the expressed levansucrase synthesizes a lethal compound, levan, from the surrounding sucrose. Thereby, the *sacB* gene, if possible, is eliminated by the cells in the presence of this sugar.

Double homologous recombination with such a plasmid occurs sequentially. In the first step, recombination takes place via the one homologous fragment (upstream) controlled by the gentamicin antibiotic selection. Thereby, the plasmid is integrated in the genome and the homologous, flanking fragments are duplicated. In this construct, both the *luxS* gene and the Gm-GFP cassette reside between the duplicated flanking regions respectively. In the second step, the recombination occurs via the other fragment (downstream) controlled by the gentamicin antibiotic and the *sacB* counter selection. Due to the gentamicin selection, the Gm-GFP resistance cassette is retained, but because of the *sacB* selection, other plasmid components are eliminated. As a result, the plasmid with the target gene is

released, and the Gm-GFP cassette remains between the flanking regions in the genome. Double homologous recombination is also known as allelic replacement, since the gene of interest is exchanged for an antibiotic resistance cassette.

Allelic replacement has been shown to occur spontaneously without a counter selection marker at 5 to 80 % frequency (Marx & Lidstrom, 2002). For example, in *Streptococcus mutans*, double homologous recombination is known to succeed without specific control. In *Sh. oneidensis*, however, only single homologous recombinants were detected without a counter selection marker. Such an event is very rare in this bacterium, therefore the usage of the *sacB* counter selection marker was necessary.

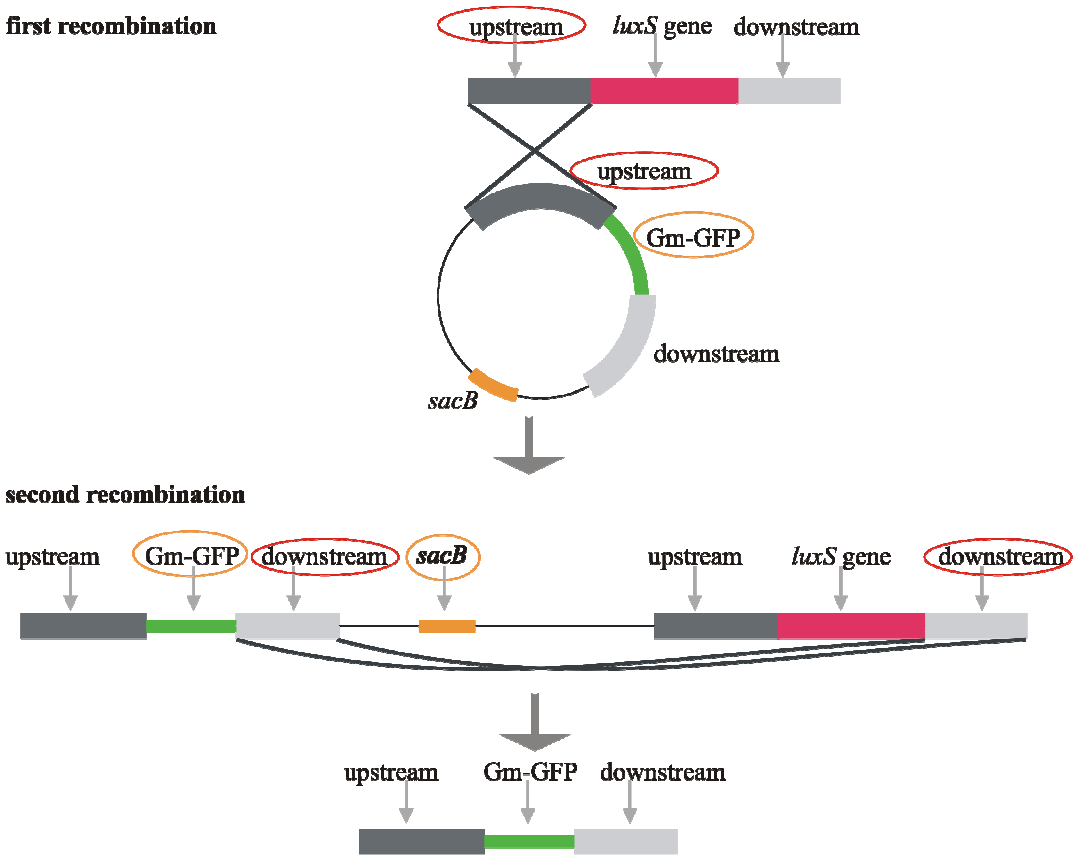


Figure 4-14. Scheme of constructing a double homologous recombination mutant in Gram negative bacteria.
The suicide plasmid contains the flanking regions of the target gene (upstream, downstream), an antibiotic resistance (Gm-GFP: gentamicin and green fluorescent protein) and a counter selection marker (*sacB*). The first recombination occurs due to the antibiotic pressure and the whole plasmid is integrated into the genome. The second recombination occurs due to the antibiotic and counter selection. Thereby the plasmid is released with the target gene from the genome and the antibiotic selection marker remains between the flanking regions in the genome.

4.4.1.3 Deletion mutant without antibiotic resistance

The removal of the Gm-GFP cassette from the double homologous recombinant was facilitated by the FLP recombinase.

FLP recombinase is a broad-host range recombinase, which originates from *Saccharomyces cerevisiae* and is able to cut out fragments flanked by the highly specific FLP recombinase target (FRT) from large DNA molecules, like genomes.

The FLP recombinase can be expressed from the plasmid pFLP2, which is not only a broad-host range plasmid but contains also the *sacB* gene, and thus it can be transferred into several species and after marker removal, it can be easily eliminated using the *sacB* counter selection.

4.4.1.4 The pKnock-Km plasmid

The pKnock-Km plasmid (Alexeyev, 1999) was used for constructing the suicide plasmids for single and double homologous recombinations (Figure 4-15). This plasmid possesses the R6Kγ *ori*, and therefore it can replicate only in strains like *E. coli* pir-116 or *E. coli* S17-1 λpir providing the Π protein encoded by the *pir* gene, but not in *Sh. oneidensis*. In *Sh. oneidensis*, it can be propagated exclusively by inserting it into the genome. In addition, the plasmid contains the RP4 origin of transfer, which enables DNA conjugation into *Sh. oneidensis*, and a kanamycin resistance marker appropriate for the selection of the pKnock-Km derivatives in *Sh. oneidensis* and *E. coli*. A plasmid with ampicillin resistance cannot be selected in *Sh. oneidensis*, since this bacterium encodes oxacillinase on the chromosome (Poirel *et al.*, 2004), and therefore it is naturally resistant to all common β-lactam antibiotics at high concentrations.

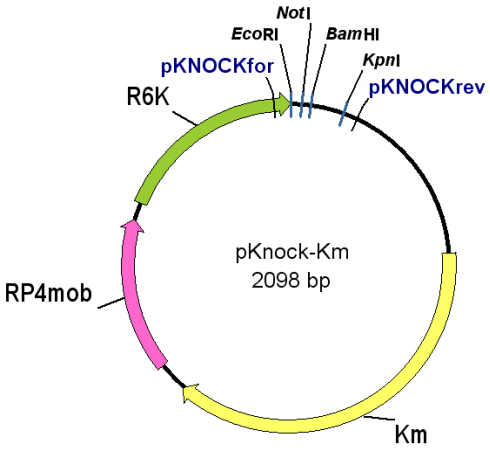


Figure 4-15. The pKnock-Km plasmid, the source plasmid for each suicide vector (Alexeyev, 1999). Km: kanamycin resistance cassette, RP4mob: origin of transfer, R6K: origin of replication, pKNOCKfor and pKNOCKrev: primers for amplification of the multiple cloning site, *Eco*RI, *Not*I, *Bam*HI and *Kpn*I: enzyme recognition sites of the multiple cloning site used in this study.

4.4.1.5 *E. coli* strains

Due to the R6K *ori*, specific *E. coli* strain that expresses the Π protein, the product of the *pir* gene, was needed to maintain the pKnock-Km derivative plasmids. *E. coli* pir-116, a genetically modified strain, fulfilled this function (Metcalf *et al.*, 1994). In this strain, a R6K *ori* containing plasmid could replicate up to 250 copies, and as a result plasmids transferred into this strain could be recovered at high yields.

To introduce pKnock-Km derivatives into *Sh. oneidensis*, conjugation techniques were employed. In the first conjugations, *E. coli* S17-1λ*pir* (Simon *et al.*, 1983) with the mobilizable plasmid, as donor, and *Sh. oneidensis*, as recipient was used in biparental matings. *E. coli* S17-1λ*pir* is a specific, multifunctional strain, since it contains the RP4 conjugable plasmid integrated in its genome enabling biparental mating and can propagate plasmids with R6K *ori*. However, this strain caused problems. Firstly, the conjugation efficiency was rather low, since only a few clones appeared after biparental matings. Secondly, the maintained and verified plasmid for double homologous recombination became modified in S17-1λ*pir* (data not shown). Conjugation and recombination into *Sh. oneidensis* was repeatedly inconclusive after biparental matings with S17-1λ*pir*. Therefore, in the later experiments, triparental matings were used with the *E. coli* HB101 strain containing the pRK2013 helper plasmid (Ditta *et al.*, 1980;Figurski *et al.*, 1979), *E. coli* pir-116 harbouring the mobilizable plasmid, as donor, and *Sh. oneidensis*, as recipient.

4.4.2 Insertional mutant and control

The *luxS*$^-$ insertional mutant, named *luxS*$^-_{ins}$, was constructed by disrupting the *luxS* gene via homologous recombination. The homologous fragment in the pKnock-Km plasmid reached from the beginning till the middle of the *luxS* gene, in order to disrupt the functional part of *luxS* in the middle. As a result of recombination, half of the *luxS* gene was located before and almost a full *luxS* gene without starting amino acids was located after the pKnock-Km plasmid insertion.

Since the antibiotic marker on the plasmid inserted in the genome could have influenced the phenotype of the cells, a control strain, named WT_{Km}, was engineered by inserting the pKnock-Km plasmid upstream of the *luxS* gene. Here, the homologous fragment was the upstream intergenic region of *luxS* extending from the middle of the *ebps* gene to the non-coding intergenic region. As a result of recombination, the upstream intergenic region of *luxS* was duplicated and the pKnock-Km plasmid was located between the duplicated fragments. The functioning of the genes was not affected.

The advantage of this method lay in its simplicity, since it consisted of one cloning into pKnock and one conjugation into *Sh. oneidensis*. However, the disadvantage was the appearance of revertants in spite of antibiotic selection (Figure 4-16) possibly due to the duplicated region in the genome.

4.4.2.1 *luxS*$^-$ insertional mutant (*luxS*$^-_{ins}$)

The suicide plasmid used for single homologous recombination was constructed using directional PCR cloning. An internal part of *luxS* between the positions of 19. bp and 213. bp was amplified with the primers LuxS_*Eco*RI_beg and the LuxS_*Kpn*I_mid. The resulting fragment, designated as *luxS*1, and the pKnock-Km plasmid were double-digested by *Eco*RI and *Kpn*I enzymes respectively. The linearized plasmid was dephosphorylated and the plasmid and the *luxS1* insert were ligated together to result pKnock-*luxS*1. After ligation, the plasmid mix was transformed into *E. coli* pir-116 for maintenance. The pKnock-*luxS*1 in *E. coli* pir-116 was selected on LB Km plates, was verified by colony PCR and restriction digestion.

Then, the pKnock-*luxS*1 plasmid was introduced into *Sh. oneidensis* wildtype by conjugation. For this purpose, the plasmid was first transformed into *E. coli* S17-1 λpir and, subsequently, transferred into *Sh. oneidensis* by biparental mating. The mating efficiency was low, but still a few *Sh. oneidensis luxS*$^-_{ins}$ clones appeared and were confirmed by PCR analysis and sequencing (Figure 4-16 and Appendix B).

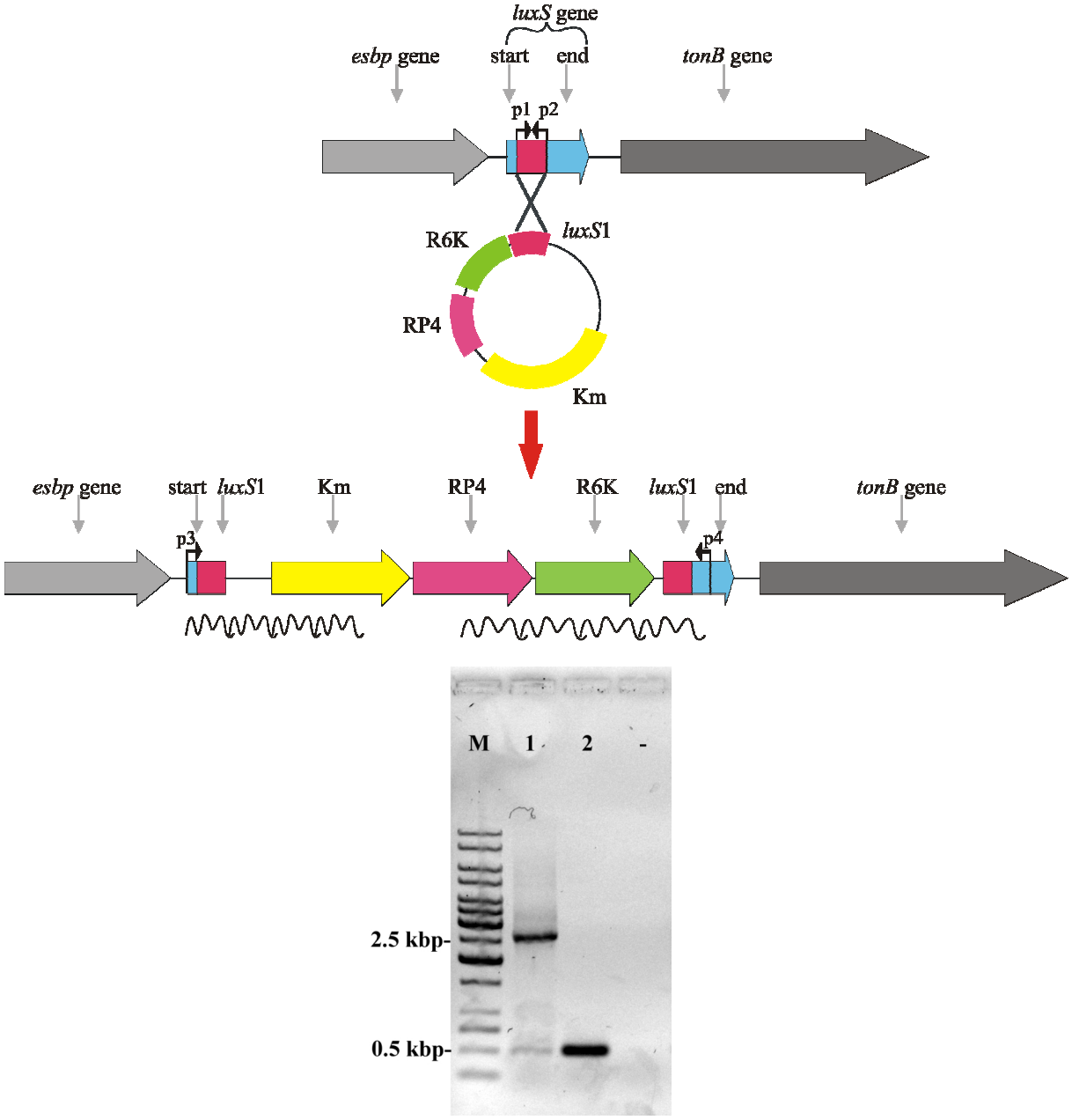


Figure 4-16 Construction and verification the *Sh. oneidensis luxS*$^{-}_{ins}$ mutant.
The *luxS1* intern fragment of the *luxS* gene was amplified by the p1 (LuxS_*Eco*RI_beg) and p2 (LuxS_*Kpn*I_mid) primers. The *luxS* gene is flanked by the *esbp* (extracellular solute-binding protein) and *tonBr* (TonB-dependent receptor). The plasmid contains *luxS1*, R6K origin of replication, RP4 origin of transfer and Km (kanamycin) resistance. The final construct contained half of the *luxS* gene, followed by the plasmid components, and the almost full *luxS* gene without the starting sequence. The final construct was verified by PCR using p3 (SonluxSfor) and p4 (SonluxSrev) primers. The sequenced segments are indicated by the wavy lines.
SonluxSfor and SonluxSrev primers amplified a 2.5 kbp fragment in the *luxS*$^{-}_{ins}$ mutant and a 0.5 bp fragment in the wildtype strain. The cloudy band at 0.5 kbp indicates the presence of revertants. M: marker, 1-4: *Sh. oneidensis luxS*$^{-}_{ins}$ mutant clones, 5, 7: *Sh. oneidensis* wildtype, *6: E. coli* S17-1 λpir/ pKnock-*luxS1*

4.4.2.2 Kanamycin insertional control (WT_{Km})

The WT_{Km} strain was constructed in the same way as the *luxS*$^{-}_{ins}$ strain. The suicide plasmid used for single homologous recombination was constructed using directional PCR cloning. A 591 bp long fragment, upstream with 17 bp spacing to the *luxS* gene, was amplified with the CluxS_*Not*I_for and BluxS_*Bam*HI_rev primers. The PCR product, named C*luxS*, and the pKnock-Km plasmid were double-digested by *Not*I and *Bam*HI enzymes respectively. The cleaved plasmid was dephosphorylated, and the plasmid and the *CluxS* insert were ligated together resulting pBA2106. The ligation mix was transformed into *E. coli* pir-116 for plasmid maintenance. The pBA2106 plasmid in the pir-116 cells was selected on LB Km plate and verified by PCR and restriction digestion.

Thereafter, pBA2106 was transformed into *E. coli* S17-1 λpir by electroporation and, subsequently, into *Sh. oneidensis* wildtype by biparental mating. Only two exconjugants appeared on selective plates, nonetheless they were also determined by PCR analysis to be the correct constructs (Figure 4-17).

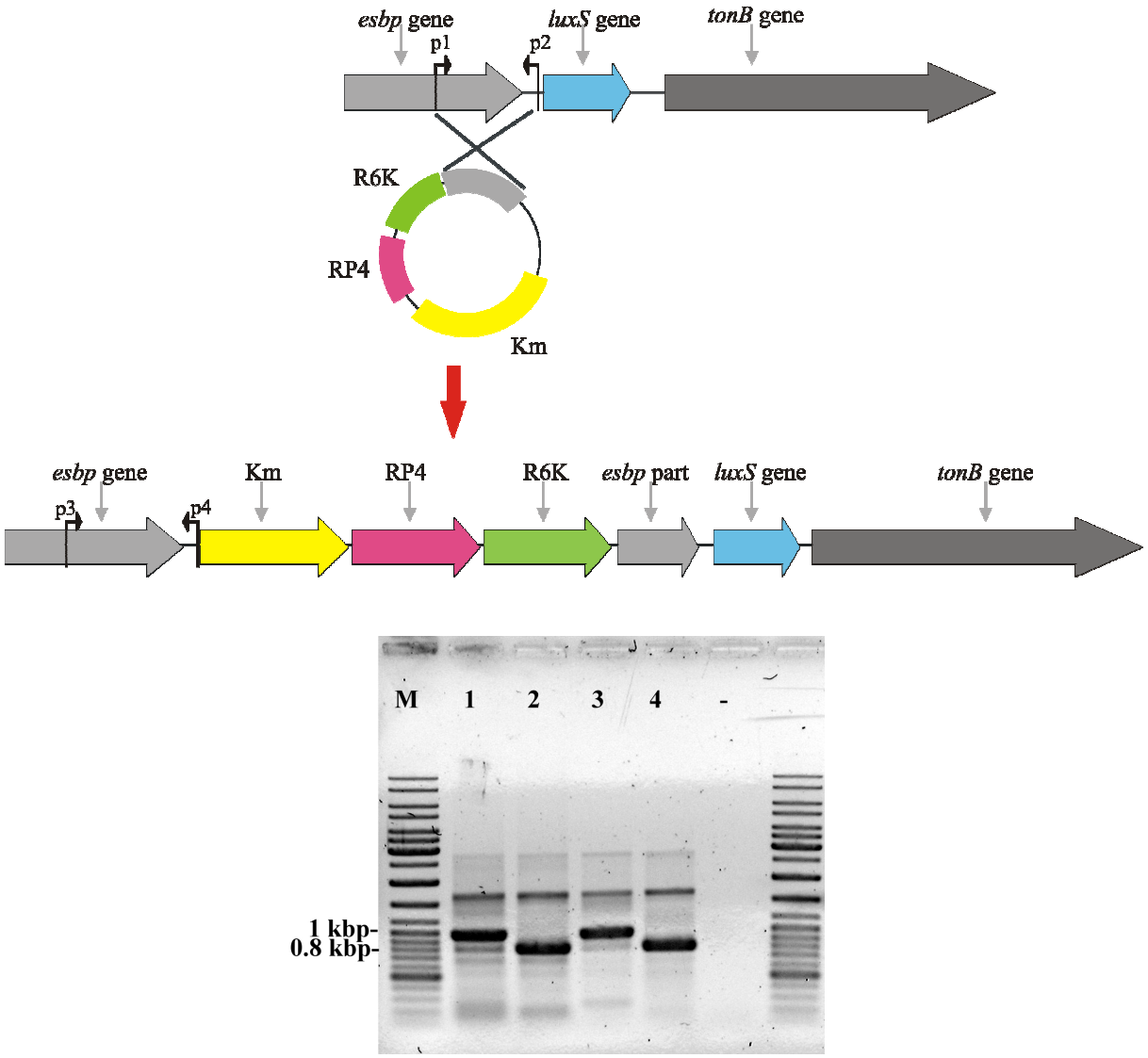


Figure 4-17 Construction and verification of the *Sh. oneidensis luxS*$^{-}_{ins}$ and WT_{Km} strain.
The upstream fragment of the *luxS* gene is cloned into the pKnock-Km plasmid resulting pBA2106. The p1 and p2 indicate CluxS_*Not*I_for and BluxS_*Bam*HI_rev cloning primers. The pBA2106 plasmid was inserted into the genome by homologous recombination. In the final construct the functioning of the genes is not affected, the plasmid is integrated before the *luxS* gene.
Verification of the WT_{Km} strain with p3 (pKnockrev) plasmid-specific and p4 (Sonfor) genome-specific primers. 1, 3: *luxS*$^{-}_{ins}$ strain, 2, 4: WT_{Km} strain.

4.4.3 Deletion mutant

The construction of the deletion mutant consisted of three steps: the construction of the allelic replacement plasmid, homologous recombinations, and the removal of the antibiotic resistance cassette from the genome. The intermediary and the final constructs were confirmed by sequencing (Sequences in Appendix C and D).

The advantages of this method were that the *luxS* gene was completely absent from the final construct, no antibiotic selection was needed to maintain the mutation and, also, the possibility of revertants was excluded. Nevertheless, the construction process was far more time-consuming than that of the insertional mutant.

4.4.3.1 Construction of the allelic replacement plasmid

The suicide plasmid was assembled in four steps (Figure 4-19). All intermediary plasmids, pBA2106, pBA13106, pBA17106, and the final pBA1147 plasmid were directly electroporated after ligation into *E. coli* pir-116 for maintenance and multiplication.

The upstream and the downstream fragments were sequentially introduced into the pKnock-Km plasmid by directional PCR cloning and their transcription directions were identical in the plasmid and in the genome. The upstream fragment, named C*luxS*, was located before *luxS* with 17 bp spacing and was 591 bp long. It was amplified with CluxS_*Not*I_for and BluxS_*Bam*HI_rev primers. The *CluxS* fragment and the pKnock-Km plasmid were double-digested with *Not*I and *Bam*HI respectively. The linearized plasmid was dephosphorylated and the plasmid and the insert were ligated together. The resulting pBA2106 plasmid in pir-116 was selected on LB Km plates.

Thereafter, the downstream fragment, *DluxS*, which was located downstream of *luxS* with 89 bp spacing and was 633 bp long, was cloned into pBA2106 plasmid. *DluxS* was amplified with AluxS_*Bam*HI_for and DluxS_*Kpn*I_rev primers. *DluxS* insert and pBA2106 plasmid were double-digested with *Bam*HI and *Kpn*I and the linearized plasmid was dephosphorylated. The plasmid and the insert were ligated together and the created pBA13106 plasmid was selected on LB Km plates.

Next, the Gm-GFP cassette from the plasmid pPS858 was inserted between the two flanking fragments of the *luxS* gene, *CluxS* and *DluxS*, into the plasmid pBA13106. The genes of the Gm-GFP unit had their own promoters, therefore the transcription direction of the cassette was insignificant and no directional cloning was used. First, the Gm-GFP fragment was removed from the plasmid pPS858 by *Bam*HI digestion and subsequent gel

purification. Then, the pBA13106 plasmid was also digested with *Bam*HI, and dephosphorylated. Finally, the Gm-GFP cassette and the pBA13106 plasmid were ligated together. The generated pBA1710 plasmid was selected on LB Gm plates.

Lastly, the *sacB* gene from the plasmid pFLP2 was inserted downstream of *DluxS* into the plasmid pBA17106. The *sacB* was excised from the plasmid pFLP2 by double-digestion with *Bam*HI and *Eco*RV resulting a 1.9 kbp, *sacB* containing fragment and a 5 kb fragment. The plasmid pBA17106 was digested by *Nhe*I enzyme. In order to produce compatible ends, the plasmid and the 1.9 kbp insert were blunted, and dephosphorylated and phosphorylated respectively. Finally, the insert and the plasmid were ligated together resulting in the plasmid pBA1147.

The final plasmid was verified by restriction digestion (Figure 4-18).

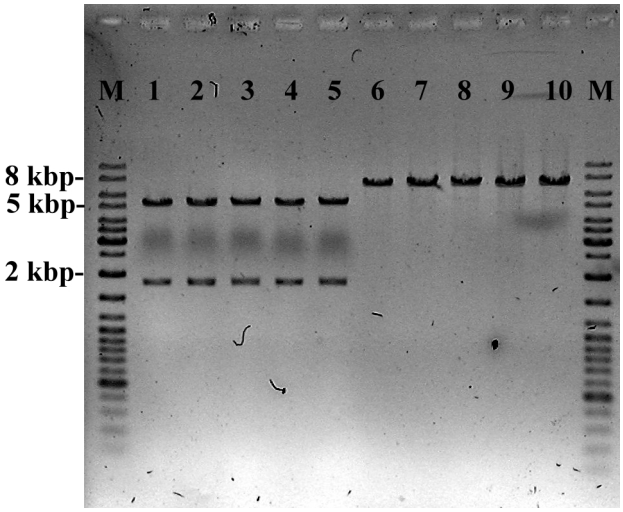


Figure 4-18. Verification of the plasmid pBA1147 by restriction digestion.
The *Bam*HI enzyme cut pBA1147 at the border of the Gm-GFP cassette resulting in a 5.2 kbp and a 1.8 kbp fragment. *Hin*dIII is a unique restriction site and linearized the plasmid resulting in a 7 kbp fragment. M: marker, 1-5: *Bam*HI digested pBA1147, 6-10: *Hin*dIII digested plasmid.

The assembling steps are illustrated in Figure 4-19.

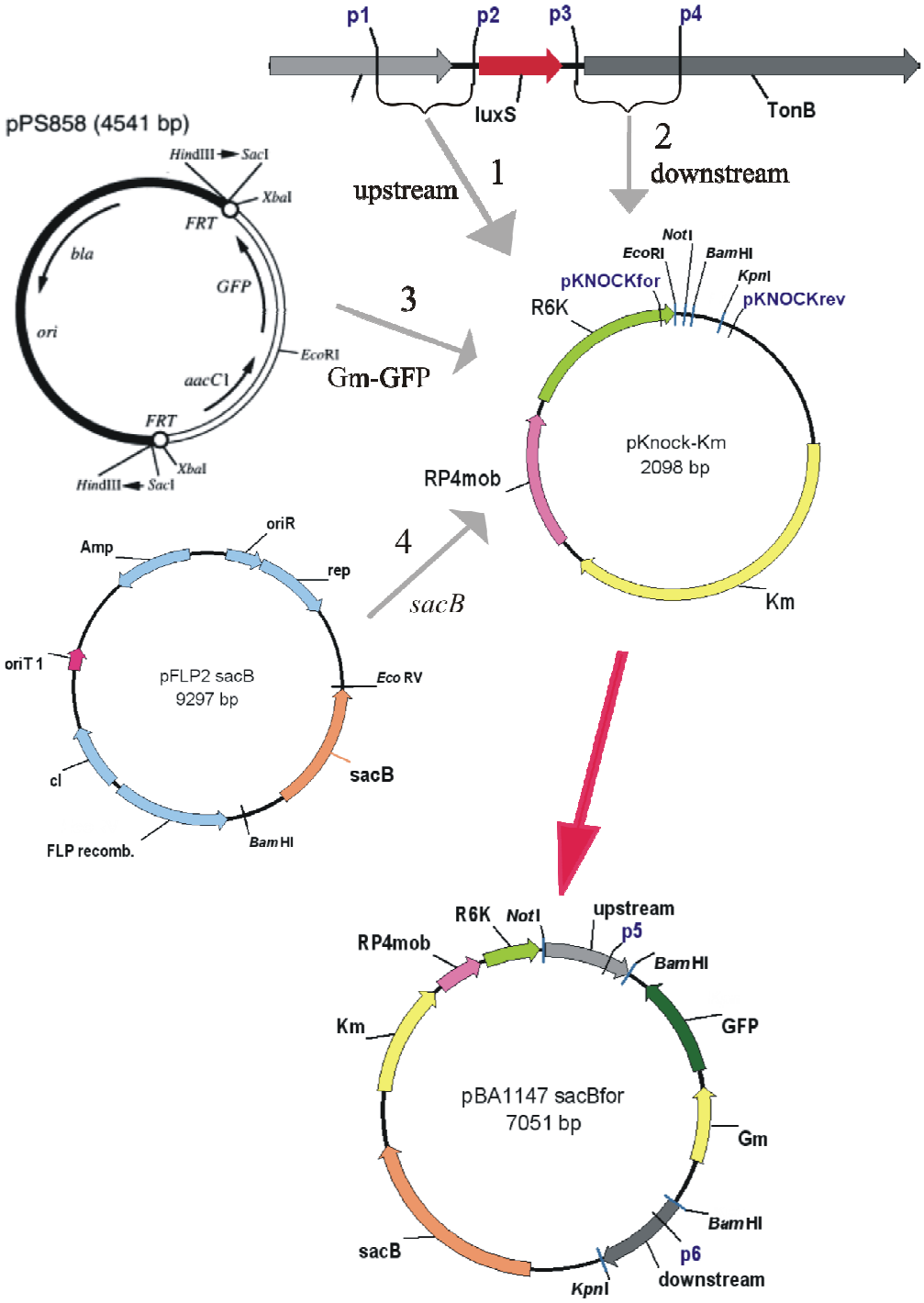


Figure 4-19. Construction of the pBA1147, allelic replacement plasmid.
First the upstream fragment amplified by p1 and p2 primers (CluxS_*Not*I_for and BluxS_*Bam*HI_rev) and the downstream fragment amplified by the p3 and p4 primers (AluxS_*Bam*HI_for and DluxS_*Kpn*I_rev) were inserted into the pKnock-Km source plasmid. Then, the Gm-GFP cassette from pPS858 was inserted between the upstream and downstream fragment using *Bam*HI restriction sites. (The figure of pPS858 plasmid is cut from (Hoang *et al.*, 1998). Abbreviations: *bla*: ampicillin resistance, *aac1*: gentamicin resistance, GFP: green fluorescence protein.) Finally, the *sacB* marker was resolved from the pFLP2 plasmid using *Eco*RV and *Bam*HI restriction sites and inserted into the allelic replacement plasmid by blunt-cloning. The insertion of the upstream, downstream fragment and the Gm-GFP cassette could be verified using p5 and p6 primers (VluxSfor and NluxSrev) or restriction digestion.

4.4.3.2 Allelic replacement

The first homologous recombination was achieved by conjugating the plasmid pBA1147 into the *Sh. oneidensis* wildtype strain using triparental mating. The exconjugants appeared after approximately one week and were analysed by the VluxSfor and NluxSrev primers. These primers bound in the middle of the upstream and downstream fragments respectively and amplified the 2.2 kb segment of Gm-GFP cassette in the integrated plasmid and the 0.9 kb segment of the *luxS* gene in the genome. If *Sh. oneidensis* inserted the plasmid via single homologous recombination, both PCR products were visible (Figure 4-21).

In triparental mating, the selection against the donor and helper *E. coli* strains was only partially sufficient. Namely, the conjugation mix was plated on SDM Gm plates, which despite lacking amino acids crucial for *E. coli* growth, did not kill the cells. According to experimental experiences, a few *E. coli* cells always survived and were usually hidden under the orange clones of *Sh. oneidensis*. After streaking the orange colonies to LB medium, the *E. coli* cells resuscitated and, in some cases, they were even sticking to *Sh. oneidensis* cells. Therefore, the appearing, orange clones had to be first streaked through several passages to obtain pure colonies and then could be verified.

The second homologous recombination was forced by *sacB* counter selection and by gentamicin antibiotic selection, i.e. the single homologous recombinant clones were transferred onto sucrose containing LB plate with Gm. The appearing clones were verified by PCR with VluxSfor and NluxSrev primers. A small proportion of the clones was determined to be a correct recombinant. These clones showed exclusively the 2.2 kb PCR band, being the amplicon of the Gm-GFP cassette on the genome and were sensitive to kanamycin. The double homologous recombinants were named *Sh. oneidensis luxS*ΔGm-GFP (Figure 4-20 and Figure 4-21). The complete sequence can be found in Appendix C.

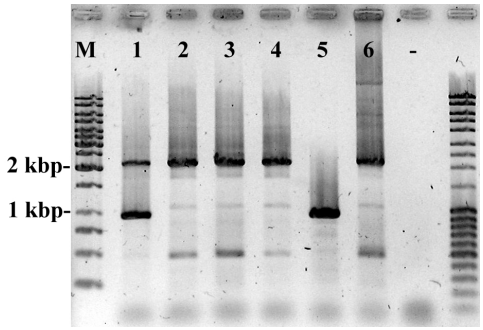


Figure 4-20. Verification of single and double recombinants.
The fragment between the upstream and the downstream fragment was amplified using VluxSfor and NluxSrev primers. If a pure *Sh. oneidensis* clone inserted the pBA1147 plasmid, both the Gm-GFP cassette (2.1 kbp) and the *luxS* gene (0.9 kbp) were amplified. In double homologous recombinants, only the Gm-GFP cassette was amplified due to the elimination of the *luxS* gene. 1: single recombinant, 2-4: double recombinants, 5: *Sh. oneidensis* wildtype, 6: *E. coli* pir-116/ pBA1147 donor strain, 7: negative control.

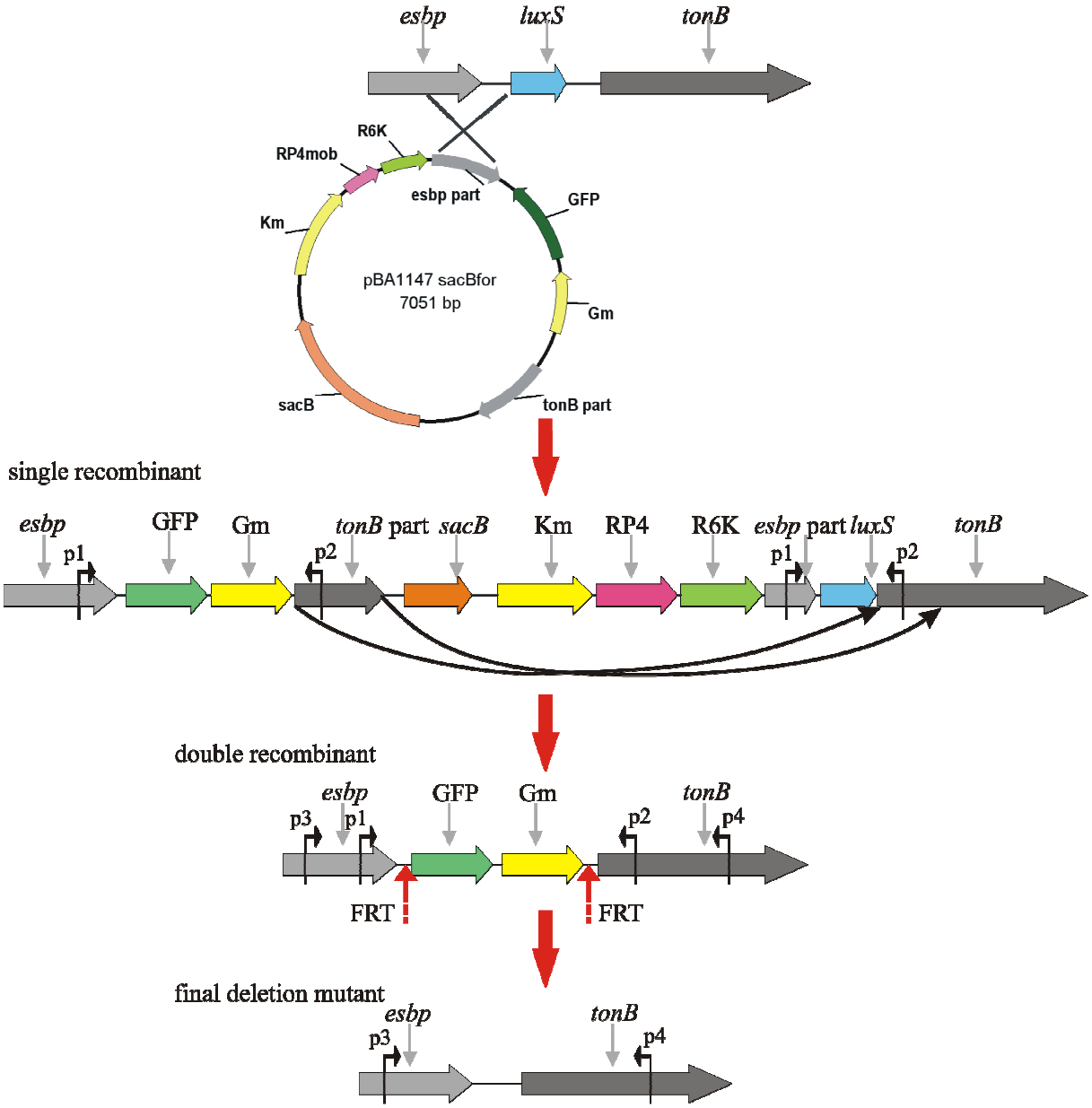


Figure 4-21. Construction of the *Sh. oneidensis* luxS$^{-}_{del}$ mutant.
First, the plasmid is inserted into the genome via crossover thus resulting in the single recombinant. Then, the plasmid with the *luxS* gene is eliminated via the second crossover thus resulting in the double recombinant. In the genome of the double recombinant, the Gm-GFP cassette remains between the two flanking genes of *luxS*, the extracellular solute-binding protein (*esbp*) and the TonB-dependent receptor (*tonBr*). Then, the Gm-GFP cassette is removed by the FLP recombinase using the FRT site thus resulting in the final, deletion mutant. In this construct, the two flanking genes of *luxS* remain in the genome.
The single and double recombinants were verified using p1 (VluxSfor) and p2 (NluxSrev) primers. The double recombinant with the Gm-GFP cassette and the final, deletion mutant were verified using p3 (Sonfor) and p4 (Sonrev) primers that bound outside of the homologous segments used for recombinations.

4.4.3.3 Removal of the Gm-GFP cassette

As the last step, the Gm-GFP cassette was removed from the chromosome of *Sh. oneidensis luxS*ΔGm-GFP using the FLP recombinase, whose recognition sites were located at the border of the Gm-GFP cassette and encoded on the pFLP2 broad-host range plasmid (Figure 4-21).

Since the plasmid pFLP2 contained only an ampicillin resistance cassette, which already exists in the genome of *Sh. oneidensis*, it should be complemented by a suitable antibiotic selection marker. The plasmid pFLP2 was cloned with the Km resistance unit from the pBSL97 plasmid (Alexeyev *et al.*, 1995) resulting pFLP2-Km.

Thereafter the plasmid pFLP2-Km was conjugated into *Sh. oneidensis luxS*ΔGm-GFP by triparental mating and the exconjugants were selected on SDM Km plates. The pure clones were analysed using the Sonfor and Sonrev primers, primers which bound outside the homologous fragments. The correct clones, missing the Gm-GFP cassette, exhibited a 1.5 kbp amplicon, while the others showed a 3.3. kbp amplicon (Figure 4-22).

Finally, the pFLP2-Km plasmid was removed from the cells. Since this plasmid also contains the *sacB* gene, it was eliminated by cultivating the clones in a sucrose containing medium.

The final construct was sensitive to both kanamycin and gentamicin and was named *Sh. oneidensis luxS*$^{-}_{\text{del}}$. The complete sequence can be found in Appendix D.

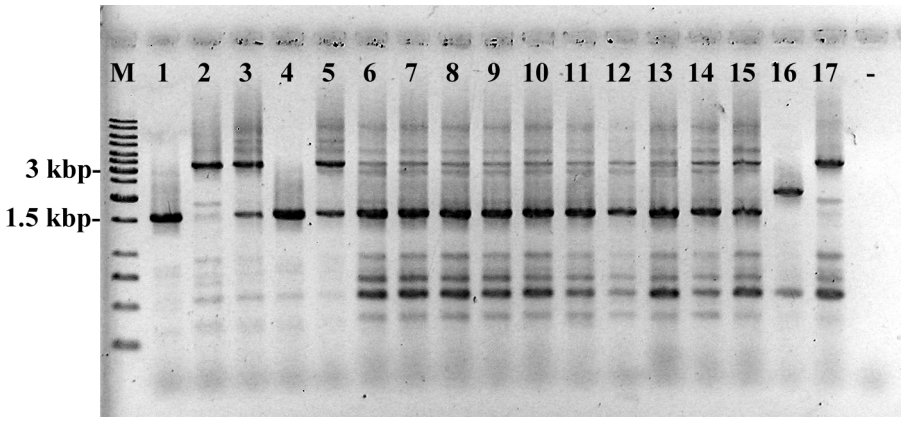


Figure 4-22. Verification of the final *Sh. oneidensis luxS*$^{-}_{\text{del}}$ mutant.
Double homologous recombinant with Gm-GFP cassette showed 3.3 kb band, while the clones of the final, deletion mutant showed 1.5 kb PCR product.
1-15: analysed clones, from which the 1. and the 4. clones are the deletion mutants. 16: *Sh. oneidensis* wildtype strain, 17: control for double homologous recombinant.

4.4.3.4 Analysis of the *Sh. oneidensis luxS*$^{-}_{del}$ strain

Analysing the transcriptional regulation of the chromosomal environment of the *luxS* gene, a mistake in the construction of the *Sh. oneidensis luxS*$^{-}_{del}$ strain was noticed. Potentially, the regulatory elements of the *tonBr* gene, the downstream gene of *luxS* were disrupted.

Namely, the *luxS* and two flanking genes were analysed to ascertain whether they form an operon. An operon is a regulatory unit in bacteria consisting of consecutive genes whose expression is controlled by a common operator and, as a result, these genes are cotranscribed into a single mRNA and translated simultaneously. The disruption of one middle-positioned gene can inhibit the transcription of the subsequent genes.

The *luxS* gene is flanked by two genes encoding the extracellular solute binding protein (*esbp*) which is 1113 bp long and the TonB-dependent receptor C-terminal region domain lipoprotein (*tonB*) which is 2052 bp long. The *esbp* gene is located upstream of *luxS* with 165 bp spacing, the *tonBr* gene is situated downstream of *luxS* with 133 bp spacing.

Although, the transcription direction of the *esbp*, *luxS* and *tonB* genes are the same, they do not form one operon. Firstly, the *esbp*, the *luxS* and the *tonB* genes do not seem to be functionally bound, since the extracellular solute-binding protein is responsible for the uptake of polyanions, the LuxS enzyme participates in the activated methyl cycle, and the TonB receptor is typically involved in the iron acquisition. Secondly, their spacing region is relatively long, while genes on one operon tend to stay in close proximity and, and their start and stop codons could even overlap (Salgado *et al.*, 2000). Thirdly, potential recognition sites for the sigma-70 transcription factor before *luxS* and *tonBr* were found manually. Before the *luxS* gene, a GATAAT sequence at -18 position similar to TATAAT Pribnow box and a GCGACA sequence at -42 position similar to the TTGACA -35 element were found. Before the *tonBr* gene, CAAAA sequence at -22 position as -10 element and ATAACA at -46 position as well as TTTACA at -54 position as -35 elements could be identified. Fourthly, probable Rho-independent transcriptional stop codon was identified by Riboswitch Explorer (Abreu-Goodger & Merino, 2005) after the *luxS* gene. Analysing the region manually, the intrinsic stop codon immediately after *luxS* can be also observed. It is a 52 bp fragment, that contains 52 % GC-residues compared to the genomial 45 % and it is followed by 9 T residue. Consequently, *esbp*, *luxS* and *tonBr* should transcribe independently.

In the course of this analysis, it was remarked, that the cloning forward primer bound very close to the *tonB* gene, and thereby the downstream homologous fragment did not contain the whole intergenic region before *tonB*. Since the Gm-GFP cassette resided immediately

before the downstream fragment in the recombination plasmid, the absent segment of the intergenic region and the *luxS* gene were eliminated in the deletion mutant. The remaining 46 bp of the intergenic region before the *tonB* gene lacks the potential -35 bp element at the -54 bp position.

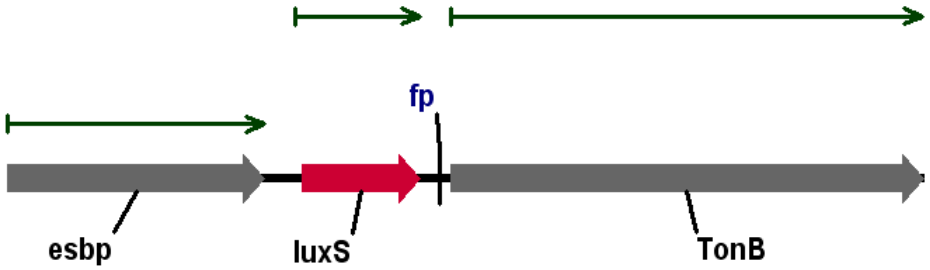


Figure 4-23. The genomic environment of *luxS* in *Sh. oneidensis*.
The *esbp* gene is on a different open reading frame to the other two genes and should be transcribed independently of them. Due to several reasons, also the *luxS* and the *tonB* genes do not form one operon. The forward primer (fp) to amplify the downstream flanking region of the *luxS* gene was very close to the *tonB* gene, therefore the regulatory elements before *tonB* are potentially disrupted.

4.4.4 Phenotypic characterization of the *Sh. oneidensis luxS* mutant

All investigations were undertaken using the *luxS*$^{-}_{ins}$ mutant and the WT_{Km} strain. The strains were compared for growth, AI-2 production, AI-2 depletion, expression of secretory proteins, biofilm growth and siderophore production.

4.4.4.1 Growth and AI-2 production

Knockout mutation of the *luxS* gene has eliminated the ability to produce AI-2 in bacteria, but usually caused no lowered growth-rates.

Sh. oneidensis luxS$^{-}_{ins}$ and WT_{Km} strains grew at an almost identical growth-rate in a LB medium, therefore all subsequent investigations were conducted simultaneously (Figure 4-24). The level of the AI-2 production of the *luxS*$^{-}_{ins}$ mutant decreased to the background level, i.e. 2 % of the positive control, while that of the WT_{Km} strain was 18% to 66% of the positive control as in the wildtype. The pattern of AI-2 production of the WT_{Km} strain was also identical to that of the wildtype. Namely, AI-2 activity increased in the logarithmic phase, and then decreased to zero level in the transition to the early stationary phase (Figure 4-24). In conclusion, the *luxS*$^{-}$ insertion mutation in *Sh. oneidensis* had no affect on growth in a LB medium but disrupted AI-2 production. The WT_{Km} strain with the *luxS* gene intact represented AI-2 production similarly to the wildtype strain.

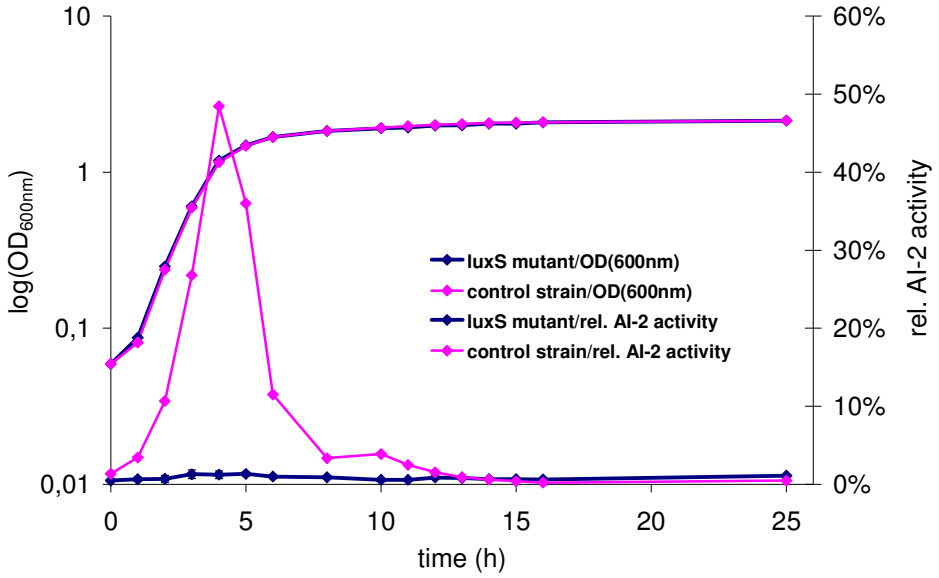


Figure 4-24. Growth and AI-2 production of *Sh. oneidensis luxS*$^{-}_{ins}$ and WT_{Km} strains.
Growth of the *luxS*$^{-}_{ins}$ and WT_{Km} strain were identical. In the *luxS*$^{-}_{ins}$ strain, AI-2 production was disrupted, whereas the AI-2 production of the WT_{Km} strain was similar to the wildtype strain. Namely, AI-2 accumulated in the exponential phase, and then declined to zero level in the transition to early stationary phase. The diagram illustrates one representative result.

4.4.4.2 AI-2 depletion by the *Sh. oneidensis* strains

As described previously, the pattern of AI-2 activity of *Sh. oneidensis* was very characteristic: accumulation in the exponential phase, peak at the transition phase and disappearance in the early stationary phase. This pattern is similar to that observed with *S. typhimurium and E. coli*. In these *Enterobacteria*, AI-2 disappears, because AI-2 is internalized by an ABC-transporter and then degraded within the cell, for which processes a designated operon is responsible. In *Sh. oneidensis*, however, no analogous operon exists and there must be an alternative cause for AI-2 depletion.

In the following investigations, the depletion of added DPD of the *Sh. oneidensis* wildtype, *luxS*$^{-}_{ins}$ and WT_{Km} strains were compared with the decrease of the endogenously produced AI-2 of the wildtype strain. Their DPD depletion indicated the timing and the specificity of the mechanism. Also, the DPD decrease of the separated cells and supernatant of *Sh. oneidensis* wildtype were compared, to clarify if the mechanism is a process of uptake or extracellular degradation.

Wildtype

Three simultaneous cultures of *Sh. oneidensis* wildtype strain were amended with DPD (Figure 4-25). The first was supplemented at the beginning, the second after 10 h of growth and the third was left as the untreated control. A fourth flask with sterile medium was also amended with DPD and treated in the same way as the cultures, to test the stability of DPD under the cultivation conditions.

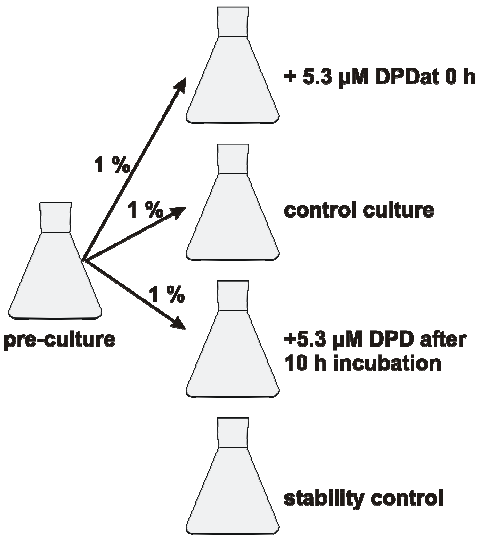


Figure 4-25. Experimental design to supplement DPD to *Sh. oneidensis*.
Three cultures were inoculated simultaneously: one was supplemented with AI-2 at the beginning of growth, another after 10 h of growth, while one more was left untreated. The negative control was sterile LB supplemented with DPD to test AI-2 stability.

If DPD was added at the beginning of cultivation (Figure 4-26), high AI-2 activity persisted in the culture for three hours, approximately till the end of the logarithmic phase. As a comparison, the untreated control had its maximum of AI-2 activity after 4 h of growth at the end of logarithmic phase. In both cultures, produced or added AI-2 was unaffected till the end of the logarithmic phase. In the transition phase, AI-2 activity of both cultures decreased precipitously and levelled off within a few hours.

If DPD was amended after 10 h of growth (Figure 4-27), the cells showed dramatic reactions. Immediately after addition, high AI-2 activity was measured, whereas 1 h after addition, almost no activity was detected.

DPD was not perfectly stable under the tested conditions (Figure 4-26 and Figure 4-27) during the whole experimental period. In each hour it declined by about 3 %. This decomposition rate is, however, still much smaller than the decrease of the cultures.

In conclusion, added DPD was removed in the same way as endogenously produced AI-2. DPD seemed not to induce the removal mechanism, since DPD added at the beginning of growth would have triggered the depletion shortly after addition. The depletion process seemed to be expressed first at the end of the logarithmic phase. It should be present in the whole stationary phase, since depletion was very rapid in this phase after 10 h of growth.

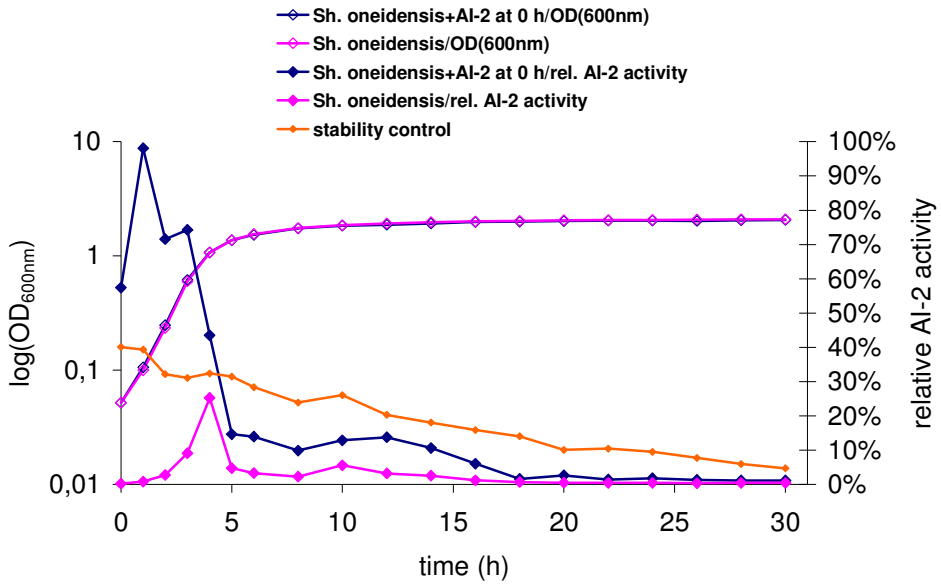


Figure 4-26. Depletion of DPD by *Sh. oneidensis* wildtype added at the beginning of growth.
DPD added at the beginning of growth (*Sh. oneidensis* + AI-2 at 0 h) was decreased by *Sh. oneidensis* first from the end of the logarithmic phase similarly to the untreated culture of *Sh. oneidensis* (*Sh. oneidensis*). DPD in the sterile medium (stability control) was somewhat decomposed under the tested conditions. The diagram shows one representative result.

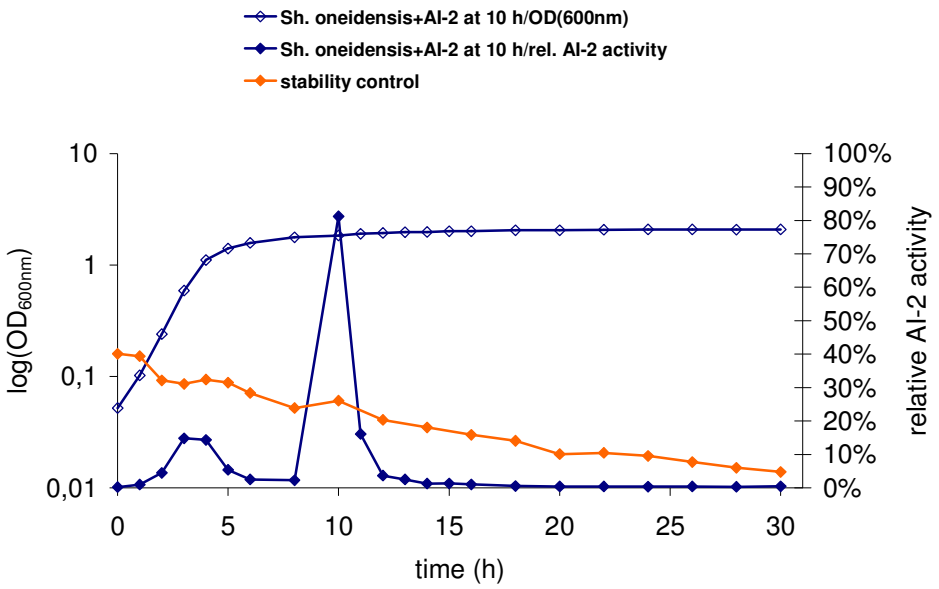


Figure 4-27. Depletion of DPD by *Sh. oneidensis* wildtype added after 10 h of growth.
Cells supplemented after 10 h of growth (*Sh. oneidensis* + AI-2 at 10 h) depleted DPD within two hours. DPD in the sterile medium (stability control) was somewhat decomposed under the tested conditions. The diagram shows one representative result.

luxS$^{-}_{ins}$ and WT_{Km} strains

Both strains were supplemented with DPD after 10 h of growth. At this growth point, the depletion mechanism was very active in the wildtype strain and no AI-2 was detected in the untreated control.

In the exponential phase, the *luxS*$^{-}_{ins}$ mutant had no AI-2 activity, while the WT_{Km} strain produced AI-2 in the wildtype manner. Nevertheless, both strains depleted DPD after 10 h cultivation just like the wildtype strain did. Namely, shortly after addition, high AI-2 activity was measured, but after 1 h, the activity diminished (Figure 4-28).

In conclusion, *luxS*$^{-}_{ins}$ mutant and WT_{Km} strains depleted DPD like the wildtype strain. Interestingly, the *luxS*$^{-}_{ins}$ mutant exhibited DPD depletion despite the lack of AI-2 in the exponential phase. This indicates that the removal mechanism is still needed in spite the lack of AI-2 and thus it should be a general mechanism, which is unspecific to AI-2.

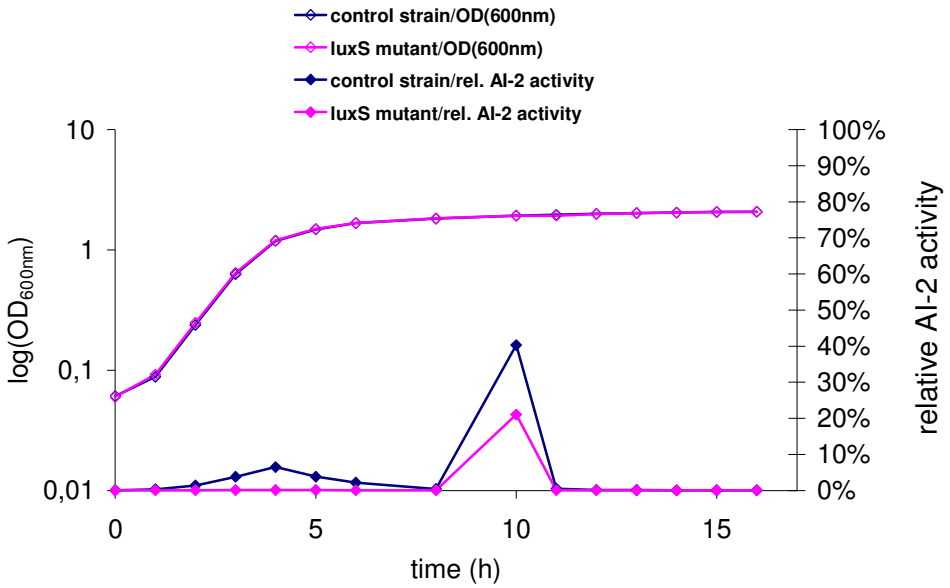


Figure 4-28. Depletion of DPD by *Sh. oneidensis luxS*$^{-}_{ins}$ and WT_{Km} strains.
Sh. oneidensis luxS$^{-}_{ins}$ had no AI-2 activity in the exponential phase, whereas WT_{Km} had AI-2 activity similarly to the wildtype. After 10 h of growth, both strains depleted the supplemented DPD within one hour.
The relative AI-2 activity values were calculated by dividing luminescence of *V. harveyi* with the culture supernatants of the *Sh. oneidensis* strains by that with AI-2 dissolved in AB medium.

Uptake or degradation of AI-2

Two processes can be responsible for the mechanisms of AI-2 depletion: uptake by the cells and extracellular degradation in the supernatant. To answer this question, the cells and the supernatant of *Sh. oneidensis* wildtype strain were independently supplemented with DPD and investigated for AI-2 removal (Figure 4-29).

After 10 h growth, the cells were pelleted and resuspended in PBS buffer, and the supernatant was sterilized. The solutions were halved, from which the one part was left untreated and the other part was heated at 95 °C for 20 minutes to inactivate potential enzymes causing AI-2 disappearance. After adjusting the solutions to the cultivation temperature (30 °C), DPD was added to each solution and to sterile PBS buffer and sterile LB medium that served as control for the cell suspension and the supernatants respectively.

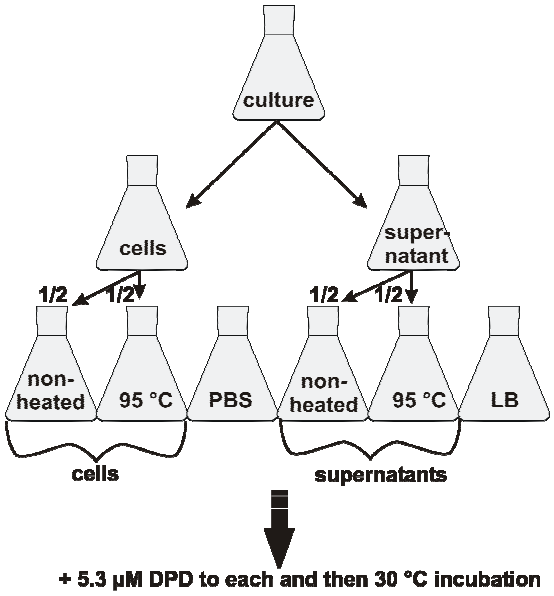


Figure 4-29. Uptake or degradation: experimental design.
After 10 h growth, the cells and the supernatant were separated by centrifugation (4000 x g, 6 min, RT). The cells were resuspended in PBS buffer, the supernatants were sterilized by filtration. Both solutions were halved, and one half of the cells and the supernatants was heat-inactivated at 95 °C for 20 min. Sterile PBS buffer and LB medium of the same volume were the control solutions. All six solutions were supplemented with DPD, and then incubated at 30 °C.

The living cells were taking up AI-2 within one hour (Figure 4-30). In contrast, the heat-killed cells left AI-2 unaffected throughout the course of the experiment. Therefore, the cells should indeed internalize AI-2, and this uptake appears to be so rapid that it should be undertaken by an active, energy-driven mechanism.

In the supernatants AI-2 activity declined too (Figure 4-30). The rate of the AI-2 decrease was slower compared to the uptake by the cells, nevertheless, still substantial, since only a small proportion of AI-2 activity was detected after 1 full hour of addition. No difference in AI-2 removal was determined between the heat-inactivated and non-treated supernatants, therefore the agent was not disrupted by 95 °C and it might not be a degrading enzyme.

Finally, AI-2 stability in the LB medium and PBS buffer was investigated under the experimental conditions (Figure 4-30). AI-2 activity was stable in the course of this experiment. This observation seems to contradict the results described in the previous section, nevertheless it should be noted that this experiment lasted for 4 hours, while the other experiment for 30 hours.

In conclusion, AI-2 depletion by *Sh. oneidensis* can be partially caused by AI-2 uptake by the cells and partially by extracellular, non-enzymatic processes.

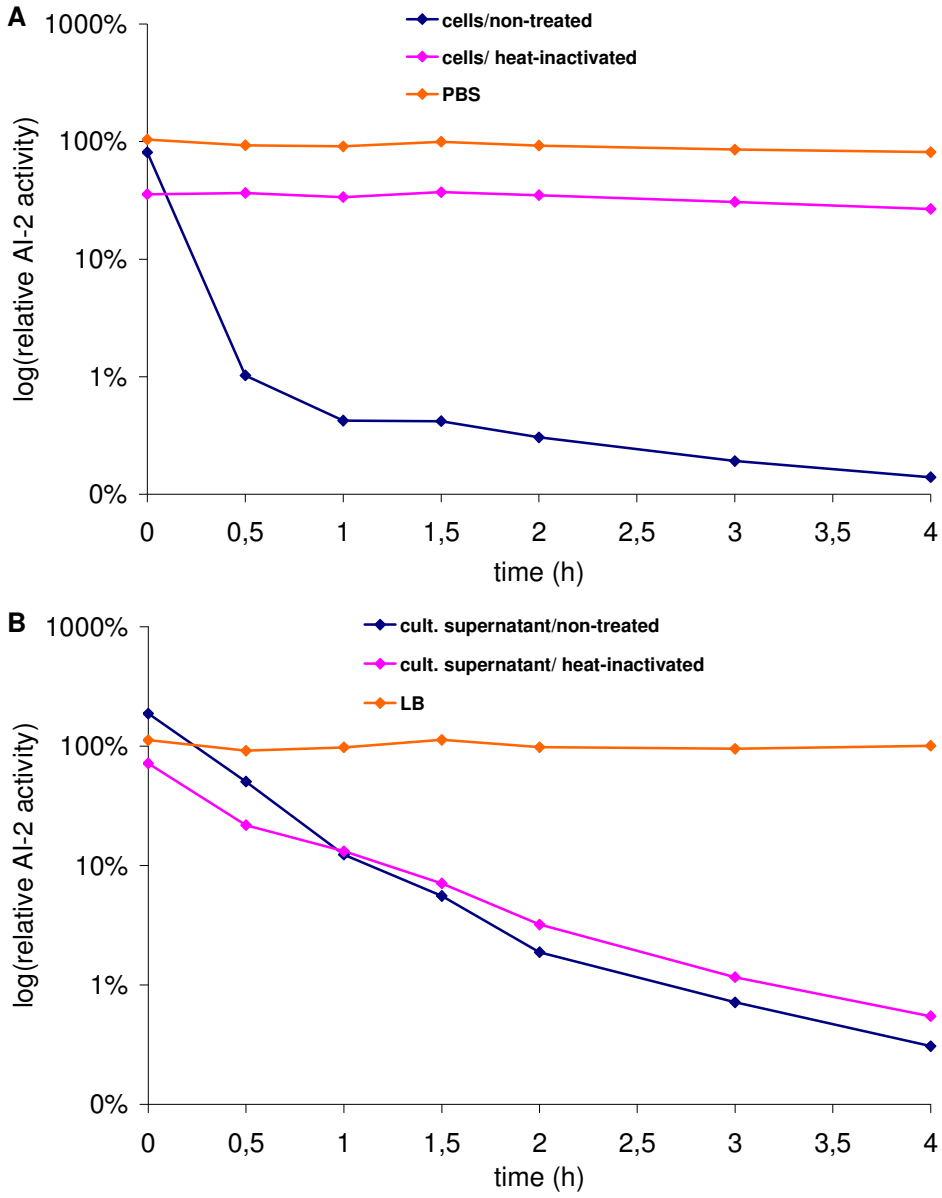


Figure 4-30. DPD depletion by separated *Sh. oneidensis* cells and supernatants.
A: The non-treated *Sh. oneidensis* cells removed supplemented DPD rapidly, while the heat-killed cells did not. In the PBS buffer, DPD was stable under the tested conditions during the course of the experiment.
B: AI-2 activity decreased in the non-treated as well as in the heat-inactivated supernatant. In contrast, DPD was stable in LB medium during the course of the experiment.
In both diagrams logarithmic scale was used in order to alleviate the high standard deviation between the samples and within one sample. The diagrams show one representative result.

4.4.4.3 Secretome analyses

The strains were cultivated in LB medium and harvested for protein extraction at OD_{600nm}=1.5. At this point, AI-2 level was regularly in the decreasing phase. It was presumed that any secreted protein, which could be regulated by AI-2, had already been expressed and should be present in quantitable amounts.

The peptides solutions of the *luxS*$^{-}_{ins}$ and the WT_{Km} strains were differentially labelled by iTraq reagents and analysed using liquid chromatography combined with tandem mass spectrometry.

The experiment was repeated twice. 172 proteins and 169 proteins were identified in the first and in the second experiment, respectively. The identified and quantitated proteins of the two replicas were sorted according to whether they existed only in one or in both secretomes. 105 were common, 67 and 64 occurred only in the first and second test respectively. In conclusion, both times sufficient number of proteins were identified and a substantial proportion of them were found again the second time, too.

The score gave general information about the identification quality, i.e. about the numbers of the unique peptides assigned to a single protein. The best score was occupied by RTX toxin, which must be the main component of the *Sh. oneidensis* secretome.

Several protein hits, like 60 kDa chaperonin, 50 S ribosomal proteins, appeared to be non-extracellular. Presumably, a part of the cell community was lysed thereby causing the appearance of intracellular proteins in the secretome. Using the PsortB online tool, non-extracellular protein hits could be picked out.

First, the up- and downregulated proteins were identified using the 2 and 0.5 fold regulations as a cut-off limit respectively. From the two lists, four proteins were identified (Table 4-3) generally indicating marginal change due to the *luxS*$^{-}$ mutation. The fist protein is a hypothetical protein being upregulated in the one sample. It is a genus-specific protein, since the best homologies were exhibited by the *Shewanella* species, although lower homologies were found of hypothetical proteins of *Xanthomonas* species, too. The second and third proteins were expressed lipoproteins and were downregulated in the one sample. These were homologous to several secreted proteins, like PKD domain containing protein, type I secretion protein, putative RTX toxin. The last, periplasmic nitrate reductase was triggered in one sample and has a role in anaerobic respiration using nitrate. All proteins except the periplasmic nitrate reductase were extracellular according to PsortB. However, these different expressions were not replicable, since they occurred only in the second

sample. In the first sample, they were either not regulated or not expressed. In conclusion, they cannot be differentially regulated in the *luxS*⁻ mutant.

Table 4-3. Proteins differentially regulated in the *luxS*⁻$_{ins}$ strain.

Name	Swiss-prot no.	Score 1	Regu-lation 1	Score 2	Regu-lation 2
Hypothetical protein	Q8EJB3	36	1,16	111,12	2,23
Expressed lipoprotein	Q8E833	26	1,00	70,89	0,17
Expressed lipoprotein	Q8CMJ0	26	1,00	70,89	0,17
Periplasmic nitrate reductase	Q8EIJ1	-	-	104,23	2,02

Second, proteins were identified, which were common to both lists and showed a trend for differential regulation, i.e. minimum 1.2 and maximum 0.8 fold regulations. The relaxation of the cut-off limits were justified by high sensitivity of the method.

Seven such proteins were identified (Table 4-4). Two components related to motility, flagellin and MshA (mannose-sensitive hemaglutinin) pilin protein were slightly upregulated in the *luxS* mutant. The flagellin is a major component of the flagella. The MshA pilin constitutes the type IV pilus, which mediates twitching motility. Three metabolic enzymes, as cysteine synthase, malate dehydrogenase and transketolase, were also slightly upregulated. Furthermore, two hypothetical proteins were slightly downregulated. One of the two hypothetical proteins, Q8EAF5, displayed homology to toluene tolerance family proteins (Pfam search: Toluene tolerance Ttg2, 1.2e-52). The other protein exhibits a putative zinc-binding motif (Prosite) and low homologies to D-aminopeptidase in its C domain (Pfam, 0.87). A similar protein is, for example, VanX that is present in several bacteria, it is also a zinc-binding aminopeptidase and was shown to function potentially for antibiotic resistance and/or survival (Lessard & Walsh, 1999). Also, a D-aminopeptidase in *Ochrobactrum anthropi* confers resistance against β-lactam antibiotics (Bompard-Gilles *et al.*, 2000). Therefore, this protein of *Sh. oneidensis* might confer resistance against antibiotics. All these proteins are membrane-associated or their position cannot be determined, and therefore they are potential secretory proteins.

Table 4-4. Proteins showing trend for differential expression in the *luxS*$^{-}_{ins}$ strain.

Name	Swiss-prot no.	Score 1	Regu-lation 1	Score 2	Regu-lation 2
Flagellin	Q8ECA6	28	1,36	171,53	1,21
MSHA pilin	Q8EA04	47	1,15	610,14	1,24
Malate dehydrogenase	P82177	79	1,20	389,31	1,53
Cysteine synthase	Q8ED64	295	1,26	483,77	1,37
Transketolase	Q8EIB3	80	1,31	178,75	1,78
Hypothetical protein	Q8EAF5	68	0,76	69,61	0,80
Hypothetical protein	Q8EJQ8	186	0,71	832,3	0,75

In conclusion, the *luxS*$^{-}_{ins}$ mutant showed a marginal change in the secretome. The main components of the secretome, as the RTX toxin, were not differentially regulated in the *luxS*$^{-}_{ins}$ mutant. Potentially, two motility-related proteins and metabolic enzymes were upregulated, while proteins that might confer resistance against aromatic solvents and antibiotics were downregulated.

4.4.4.4 GFP-tagging of the test strains

To enable microscopic documentation of living bacteria, the *luxS*$^{-}_{ins}$ mutant and the WT_{Km} strain were labelled with GFP by introducing the pEX18ApGm plasmid.

The pEX18 plasmids can replicate in *Sh. oneidensis* due to the *Col*E1 *ori* (Claribel Cruz-García, personal communication). The pEX18ApGm plasmid resulted from the ligation of the pEX18Ap plasmid and the Gm-GFP cassette from pPS858. The Gm-GFP cassette complemented the plasmid with gentamicin resistance cassette suitable for *Sh. oneidensis* and with a constitutively expressed GFP gene. Due to the GFP expression plasmid, both strains exhibited bright green cells.

4.4.4.5 Biofilm growth monitoring

The biofilms were grown in a batch system on microscope glass slides in a Petri dish filled with defined minimal medium (SDM) for four days under aerobic conditions at 30 °C. At each developmental point, one dish of each strain was removed and representative locations of individual biofilms were imaged using a fluorescence microscope (Figure 4-31). As soon as three-dimensional structures were visible, the biofilms were documented also using a confocal laser scanning microscope (CLSM) (Figure 4-32). The biofilms recorded by CLSM were quantified using the Phlip toolbox, and two parameters, biovolume and substratum coverage were analysed as the function of time (Figure 4-33). The thickness of the biofilm was estimated using the Imaris software.

Both the mature structure and the dynamic of the *luxS*$^{-}_{ins}$ and WT_{Km} biofilm had discrepancies. The mutant represented less differentiated, well-covering mats of loosely-bound cells, while the control tended to gather in tight, very flat and round-shaped clusters. In addition, the *luxS*$^{-}$ mutant biofilm developed faster and after four days it was already in the detaching phase, while the control was still developing.

At the early developmental stages, up to 9 h, both strains displayed a homogenous single layer that grew denser gradually. The mutant and the control were indistinguishable.

After 19 h, both strains formed the first elementary aggregates and the single layer between the clusters looked thinner than observed previously. The cells might have become detached at some locations, while sticking together at the others. At this stage, the mutant and the control exhibited the first alterations. Namely, the mutant had a denser single layer than the control and the mutant exhibited a homogenous culture, while the control cells looked different in length ranging from short vestiges to normal rods.

After 29 h, the mutant displayed a dense layer with locational aggregates, while the single layer of the control remained thin and only the clusters had become wider.

After 43 h, the differences between the mutant and control had become more apparent. The *luxS*$^{-}_{ins}$ mutant was homogenously distributed on the surface and formed an even, porous layer whereas the control exhibited compactly packed, highly symmetric, round-shaped and very flat structures, and between them, large voids were visible. The height of the mutant biofilm was twice that of the control biofilm being approximately 15 to 20 µm and 7 µm respectively. The different appearance was reflected by Phlip parameters, as well. The substratum coverage of the mutant was 49 %, while that of the control only 19 %. The biovolume of the mutant was three times that of the control.

After 51h and 68 h incubation, the appearance of the mutant remained similar, while the compact clusters of the control were wider and eventually fused; and the control also formed a dense homogenous layer in a few places. The heights of the mutant and the control biofilm increased and reached 25-29 µm and 10-14 µm respectively. According to the Phlip analyses, the biovolume and the surface coverage of the mutant biofilm remained at around the same level, while that of the control biofilm increased. Hence, the mutant biofilm appeared to have reached its maximum developmental stage after 43 h incubation and remained at this level for one more day, while the control biofilm was growing during the whole period.

After 93 h, the mutant biofilm became more porous and seemed to have arrived at the detachment phase. The control biofilm was still growing. The height of the mutant biofilm was halved to 16 µm and its biovolume and substratum coverage dropped also indicating that the mutant had been detached, whereas the height of the control biofilm grew to 16-19 µm and, also, its biovolume and the substratum coverage had increased.

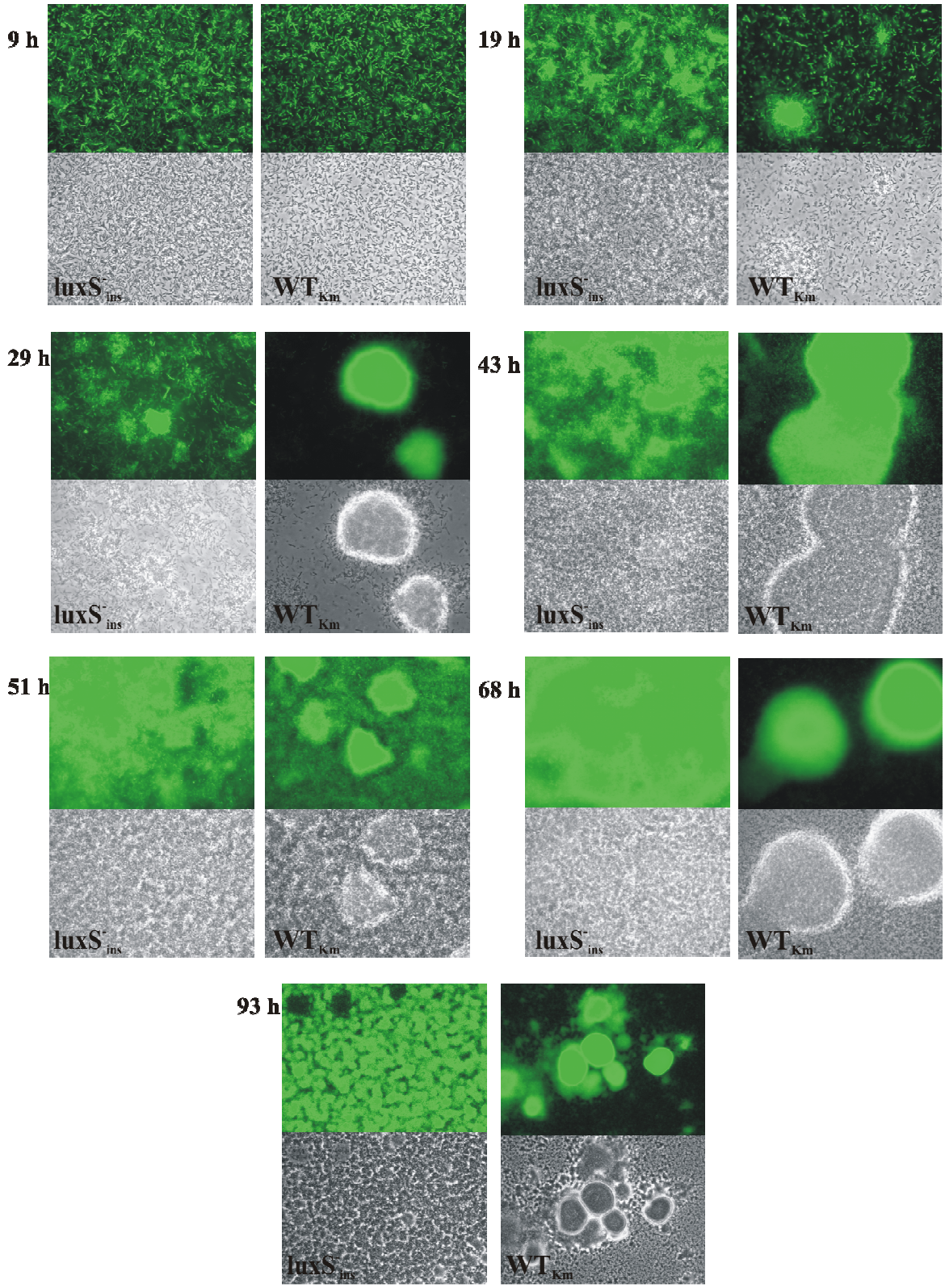
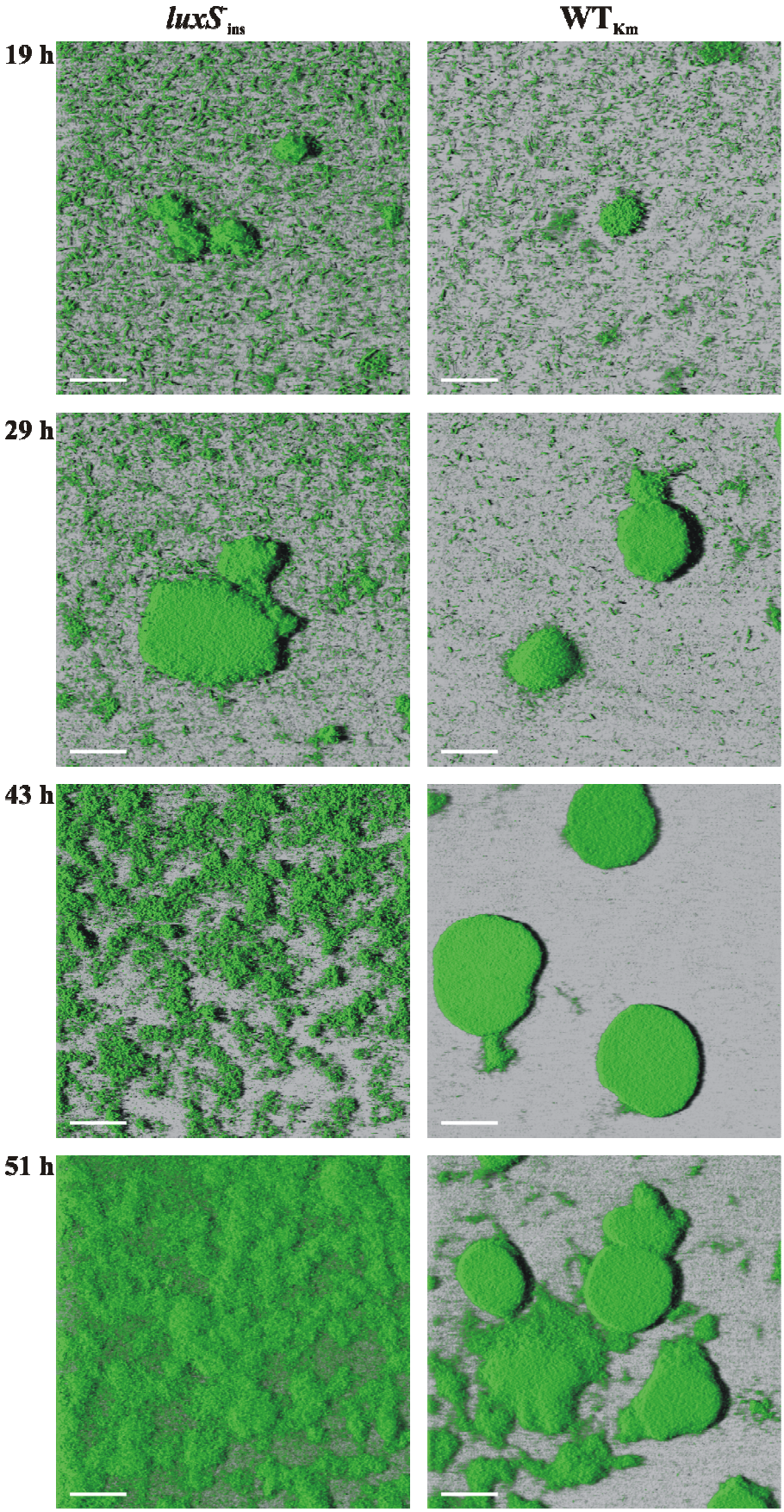


Figure 4-31. Biofilm development of *Sh. oneidensis luxS*$^{-}_{ins}$ and WT_{Km} on epifluorescence images. In the first hours till about 10 h of incubation, single layers of both strains were visible. After 19 h growth, the first aggregates were documented. From 19h till the end of the experiment, the *luxS*$^{-}_{ins}$ formed a less-differentiated biofilm than the WT_{Km} strain which tended to group into tight clusters. The micrographs are selected from 2-5 records. The images of 9 to 68 h developmental points were documented using 40x magnification, that of the 93 h biofilm were documented with 10x magnification.


luxS ins
WT Km
19 h
29 h
43 h
51 h

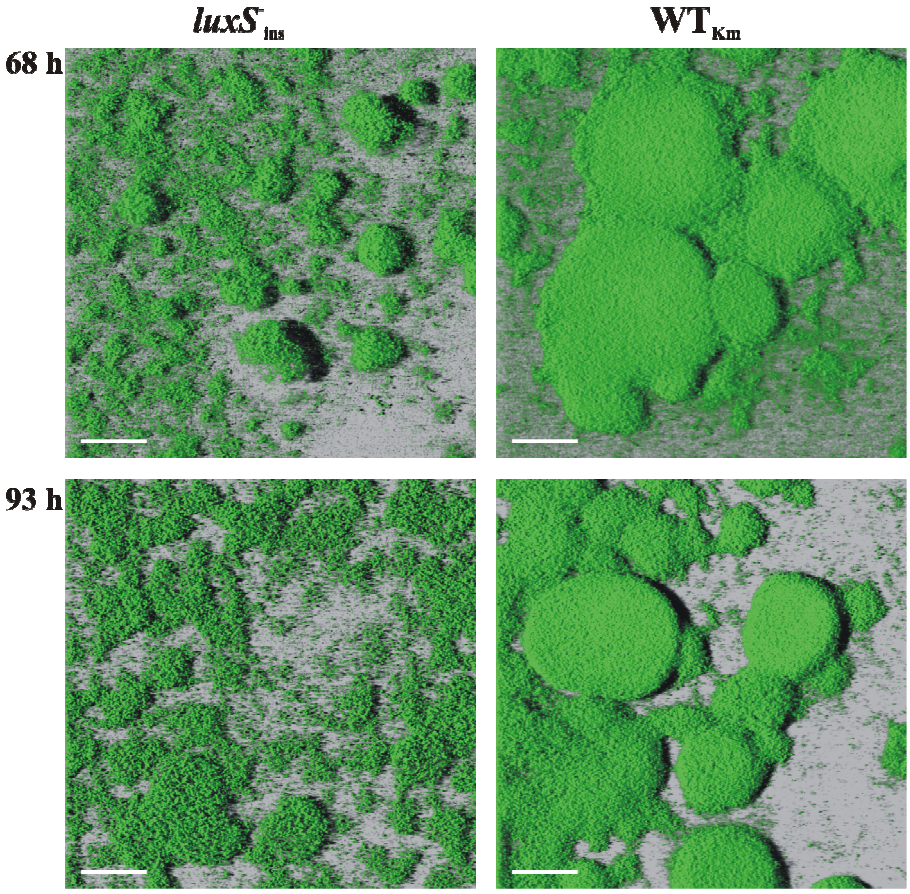


Figure 4-32. Biofilm growth of *Sh. oneidensis luxS*$^{-}_{ins}$ and WT_{Km} strains on CLSM micrographs.
The first three-dimensionally recordable structures appeared after 19 h incubation. The *luxS*$^{-}_{ins}$ strain covered the surface with loosely bound cells, while the WT_{Km} strain clustered into round aggregates. From 43 h till 68 h, the *luxS*$^{-}_{ins}$ biofilm was also higher than the biofilm of the WT_{Km} strain. After 93 h incubation, the *luxS*$^{-}_{ins}$ biofilm was already in the detachment phase, while the WT_{Km} biofilm was still in the attachment phase. The images are one of two records. The scale bars indicate 50 µm.

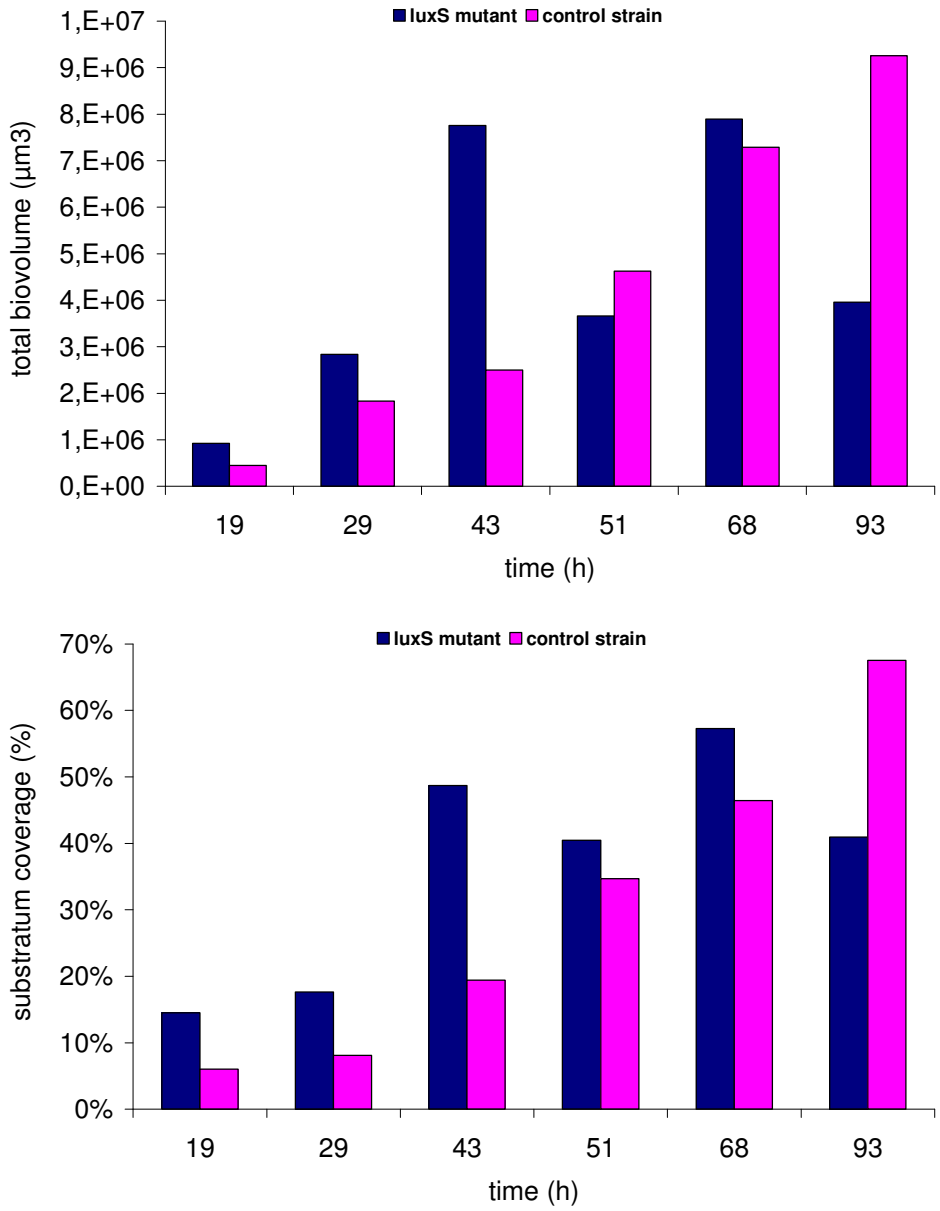


Figure 4-33. Biovolume and substratum coverage of the biofilms of *luxS*$^{-}_{ins}$ and WT_{Km} strains.
The biovolume of the *luxS*$^{-}_{ins}$ biofilm was higher than that of the WT_{Km} biofilm after 19, 29, 43 and 68 h incubation.
The substratum coverage of the *luxS*$^{-}_{ins}$ biofilm was higher than that of the WT_{Km} biofilm till 68 h incubation.
Both biovolume and substratum coverage indicated that the *luxS*$^{-}_{ins}$ biofilm was in the detachment phase after 93 h of incubation, while the WT_{Km} biofilm increased during the whole experimental period.

In conclusion, the *luxS*$^{-}_{ins}$ mutation in *Sh. oneidensis* changed the adhesion to the surface, the mutual attachment of the cells and the stratification of the biofilm, but the mutation did not affect the initial adhesion of the cells to the glass surface.

The biofilm of the *luxS*$^{-}_{ins}$ mutant developed faster and demonstrated a different structure compared to the biofilm of the WT_{Km} strain. The faster development could be due to faster proliferation, since the mutant dishes turned into bright orange-brown colour earlier than

the WT_{Km} dishes, and this colour is characteristic to cultures of *Sh. oneidensis* in the stationary phase. The *luxS*$^{-}_{ins}$ mutant and the WT_{Km} strain formed structurally different biofilm, i.e. the cells of the *luxS*$^{-}_{ins}$ mutant connected more loosely and spread better on the surface than the cells of the WT_{Km} strain. Because of better spreading, the surface to volume ratio of the mutant biofilm was higher, and more cells were located at the frontier. Therefore, more cells were directly exposed to potential attacks and the secreted exoenzymes of the cells had more possibility to diffuse away.

4.4.4.6 Biofilm complementation experiment

A *luxS*$^{-}$ mutant can lack AI-2, as a quorum sensing signal, and metabolites of the interrupted activated methyl cycle, like homocysteine and methionine. Therefore, synthetic AI-2 and methionine were added to the biofilm of the *luxS*$^{-}_{ins}$ mutant, to test whether the observed structural change in the biofilm was caused by signalling via AI-2 or the interruption of the activated methyl cycle. The biofilm of the WT_{Km} strain was also supplemented with DPD and methionine, to control any potential adverse effects of the compounds.

To guarantee an equal number of starting cells in each biofilm dish and also because six parallel cultures were inoculated instead of two, the biofilm preparation was changed. After cultivation of the strains in LB, the pre-adhesion step in LB was omitted. The SDM medium was planktonically inoculated directly from the LB cultures to 5 % density. Then these homogenized cell solutions of the mutant and the control were split into three parts. The first solution of each strain was left untreated; the second of each was supplemented with DPD: the third of each was amended with methionine. The biofilm dishes were incubated at 30 °C, and at each analysis point one dish per strain and per treatment was removed and monitored by microscopy as detailed in the previous section.

In hindsight, the pre-adhesion step in LB could have been crucial for the success of the experiment. In fact, the difference between the *luxS*$^{-}_{ins}$ and the WT_{Km} strain observed previously could not be seen. Consequently, the treatments lost their significance.

The initial adhesion was slower in this experiment. After 18 h of incubation, a single layer and a few microcolonies were recorded and no three-dimensional structures were observed. After 28 h and 42 h, the first mounds appeared, but the mutant also formed the tight, round clusters, and the control spread even better on the slide. After 52 h and 66 h of incubation, an extensive carpet was visible on both biofilms. Both biofilms reached their maximum developmental stage after 66 h incubation, when the mutant covered the surface better than the control. After 90 h, both biofilms became porous indicating that the biofilms were at

the detachment phase. Interestingly, the maximal height of the biofilms was 50 µm, almost double as much as in the first experiment, but biofilms of both strains had similar heights.

In conclusion, the cells adhered more slowly, no structural difference between the *luxS*$^{-}_{ins}$ and the WT_{Km} biofilm could be observed and the biofilms grew simultaneously.

4.4.4.7 Biofilm formation assay

The biofilms were grown in microtitre plates, and at several time points the biomass of the biofilms was measured using the crystal violet method. In this method, crystal violet dye stains the biofilm cells. Then, the absorbed dye is extracted and the turbidity of the extracted dye (OD_{620nm}) demonstrates the biomass of the biofilm.

The biomass of the biofilms tended to be different (Figure 4-34). Already after 9 h of incubation and in the whole course of the experiment, the *luxS*$^{-}_{ins}$ mutant had higher biomass than the WT_{Km} strain, though at the beginning the opposite was true. The highest difference was measured after 24 h of incubation. Nevertheless the method had a high standard deviation, and no statistical difference could be stated.

The developmental course of the biofilms was identical, i.e. they grew simultaneously in the microtitre plates, no time delay or overtaking of the mutant biofilm could be observed (Figure 4-34).

In conclusion, the *luxS*$^{-}_{ins}$ mutant tended to form higher biomass biofilms compared to the WT_{Km} strain, but this assay result needs to be confirmed.

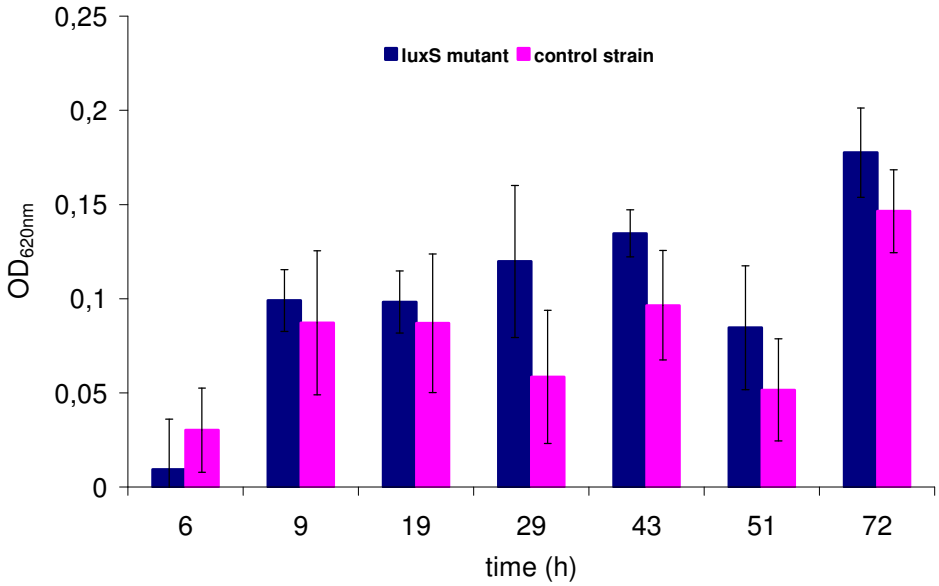


Figure 4-34. Biofilm kinetics of the *Sh. oneidensis luxS*$^{-}_{ins}$ mutant and WT_{Km} control.
The turbidity of the crystal violet stain (OD_{620nm}) indicates the biomass of the biofilms. 6 h after inoculation the WT_{Km} biofilm had a higher biomass than the *luxS*$^{-}_{ins}$ mutant biofilm, but thereafter the mutant biofilm had a higher biomass than the control biofilm at each time point. The standard deviations are indicated on the bars.

The mature biofilms in the microtitre plates were imaged by an inverse epifluorescence microscope. The structure of the biofilms formed in the microtitre plates was different from the biofilms formed on the glass slide (Figure 4-35).

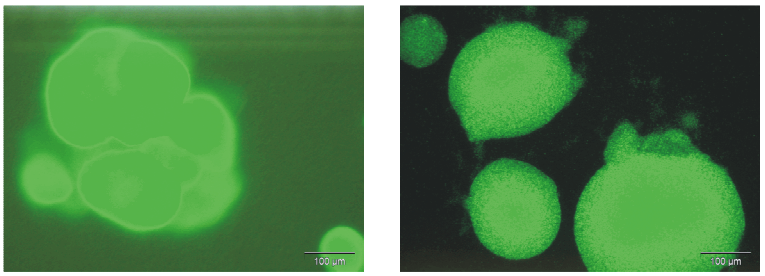


Figure 4-35. Biofilm structures of the *Sh. oneidensis* WTKm strain in the microtitre plate and on the glass slide.
Mature biofilms of the WT_{Km} strain are shown on both images. The image on the left illustrates the biofilm in the microtitre dish, while the figure on the right shows the biofilm on the glass slide. The WT_{Km} strain formed asymmetric structures in the microtitre dish, but completely symmetric compact clusters on the glass slide. Both images were recorded using 10x magnification, the scale bars indicate 100 µm length.

4.4.4.8 Siderophore production

Shewanella species (named *Sh. putrefaciens* in 1994) were found to produce hydroxamate-type of siderophores and not catecholate-type (Gram, 1994).

To measure siderophores, the structure-independent, highly sensitive chromeazurol-S test was used. In the chromeazurol-S test, the colour change of the test solution indicates the presence of siderophores. The test solution contains a blue complex of chromeazurol S/ iron(III)/ hexadecyltrimethylammonium bromide. As a siderophore removes the iron from the complex, the colour of the complex turns from blue into yellow-orange. The test solution was added to a solid defined medium, whose colour was then dark green presumably due to the ion content of the medium. Three drops of each strain was pipetted onto this test medium and the colour change was monitored.

After two days incubation, the medium with the *luxS*$^{-}_{ins}$ mutant cells looked completely different to the medium with the WT_{Km} strain (Figure 4-36). The complete medium with the mutant was bleached to a light green colour, while the plate with the control remained dark green. Also the yellow halos around the *luxS*$^{-}_{ins}$ cell carpet were wider than that of the WT_{Km} carpet. The next day, the medium with WT_{Km} strain turned also bright green, and the media with the strains were indistinguishable.

In conclusion, till two days incubation on minimal medium, the *luxS*$^{-}_{ins}$ mutant produced more siderophores compared to the WT_{Km} strain. Since this test was performed only once, confirmation is required.

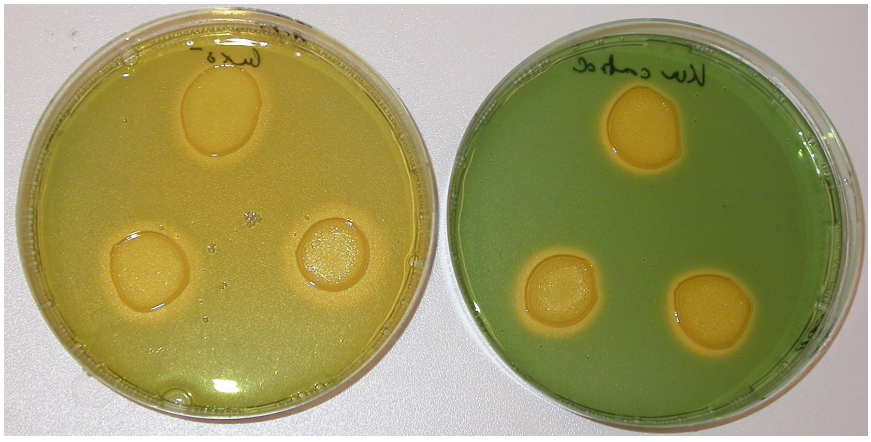


Figure 4-36. Siderophore test plates of *Sh. oneidensis luxS*$^{-}_{ins}$ mutant and WT_{Km} strain after two days incubation.
Sh. oneidensis luxS$^{-}_{ins}$ mutant (left) produced more siderophores than the WT_{Km} strain (right) after two days incubation. Larger halos around the *luxS*$^{-}_{ins}$ cells were visible and the whole plate with the mutant turned hell green. In contrast, narrow halos around the WT_{Km} cells were visible and the whole mutant plate still had the original dark green colour of the test plate

5 Discussion

5.1 Response of *P. aeruginosa* virulence genes to autoinducer-2

In a previous paper (Duan *et al.*, 2003), it was demonstrated that AI-2 might be a potential common signal between clinical isolates of *Streptococcus* species and the pathogenic *P. aeruginosa* and that AI-2 could trigger the expression of virulence genes in *P. aeruginosa* isolated from the sputum of cystic fibrosis patients.

This discovery prompted us to follow up these results and investigate the expression of the reported genes and potentially other genes in response to AI-2. The published luminescence reporter plasmid with the promoter of the *phzA1* gene (Duan *et al.*, 2003) and a GFP-reporter plasmid with the promoter of the *lasB* gene were transformed into the *P. aeruginosa* PAO_{Igl} wild-type strain, and the latter plasmid was also transformed into the *P. aeruginosa* $PAO_{lasI^-,rhlI^-}$, double mutant in a synthesis of AHL-structured autoinducers.

Although AI-2 had induced the *phzA1* and *lasB* genes 3.2 and 2.4 fold respectively in the previous study, this was not confirmed in this study. AI-2 containing culture supernatant did not induce the promoters in the *P. aeruginosa* PAO_{Igl} wildtype strain. Both AI-2 containing culture supernatant and synthetic AI-2 did not induce the *lasB* promoter in $PAO_{lasI^-,rhlI^-}$, double autoinducer synthesis mutant.

This discrepancy with the published study might be caused by the strains used in this study, which are different to the clinical isolate previously. Namely, *P. aeruginosa* is known to be an extremely adaptable species (Martin Schuster, personal communication). The PAO wildtype strain has diversified into two strains with different characteristics simply by being handled in two different laboratories. $PAO1_{Igl}$ from the laboratory of Iglewski and $PAO1_{O/V}$ from the laboratory of Ochsner/ Vasil form differentially coloured colonies: the colonies of $PAO1_{Igl}$ were yellow, while the colonies of $PAO1_{O/V}$ were brownish. In addition, they differ in their capability of reacting to reagents, since $PAO1_{Igl}$ can be made competent by chemical treatments, while $PAO1_{O/V}$ can be transformed only by electroporation. In contrast to these high diversification capabilities, 2-3 fold induction of a reporter plasmid is minor and was therefore called “modulation”.

Although this publication by Duan is frequently cited (51 times in Scopus), the work has not been followed up by other researchers. They cite it as an example for interspecies communication between bacteria of the same niche.

Nevertheless, Duan and Surette (Duan & Surette, 2007) tested in a follow-up work whether AI-2, as an environmental factor, induces the promoters of *lasI* and *rhlI* genes in the luminescence reporter plasmid in the PAO wildtype strain. The *lasI* and *rhlI* genes were not the same virulence genes as tested previously, since they encode synthesis of C12-HSL and C4-HSL, the autoinducers of *P. aeruginosa*. Nevertheless, if AI-2 had induced these genes and thus autoinducer production, it would have indicated AI-2 triggers indirectly quorum sensing-induced genes of *P. aeruginosa* listed in the previous paper thereby serving as confirmation. However, only mucin was shown to significantly induce the *lasI* and *rhlI* genes, but not AI-2.

In conclusion, AI-2 induced minimally a clinical isolate of *P. aeruginosa*, nevertheless this could not be confirmed in this study with the PAO wildtype strain and with the double autoinducer synthesis mutant and it was not confirmed in other studies. Conceivably, *P. aeruginosa* is able to adapt to a niche, and respond to available environmental factors, such as AI-2, by triggering niche-specific genes.

5.2 Phylogenetic distribution of the Pfs/LuxS and the SahH pathways

To further our knowledge of non-pathogenic species concerning the means of AI-2 synthesis and thus potential AI-2 production-signalling, 164 phylogenetically characterized marine isolates were investigated for the distribution of the Pfs/LuxS and the SahH pathways by identifying the *luxS* and *sahH* genes.

The marine isolates belonged to three phyla: *Alphaproteobacteri*a, *Bacteroidetes* and *Gammaproteobacteria*. Within one phylogenetic group, phylum or genus, the marine isolates used one pathway consistently and exhibited phylogenetic distribution of the pathways, as in previous published research with mainly pathogenic species (Sun *et al.*, 2004) In conclusion, the phylogenetic position must determine the potential for AI-2 production of a species; the life mode and the inhabited niche influence it only slightly.

All marine *Alphaproteobacteria* and *Bacteroidetes* species had the *sahH* gene, consistent with fully-sequenced species to date and therefore these bacteria must not produce AI-2. There are only two species within the *Bacteroidetes* phylum, which represent exceptions in this regard: *Porphyromonas gingivalis* (Chung *et al.*, 2001; James *et al.*, 2006) and *Bacteroides vulgatus*, completely-sequenced bacteria. *Porphyromonas gingivalis* lives in

the oral cavity, in which most of the bacteria produce AI-2 (Frias *et al.*, 2001; Kolenbrander *et al.*, 2002). *Bacteroides vulgatus* lives in the human colon, which is inhabited by numerous enterics producing AI-2 (Kaper & Sperandio, 2005; Sperandio *et al.*, 2003). In addition, their *luxS* genes are homologous to that of *Clostridiales* (data not shown) from the *Firmicutes* division and therefore they might have acquired the *luxS* gene horizontally from this group. Although other *Bacteroides* species have been also demonstrated to produce AI-2, the *luxS* gene was not identified in them (Antunes *et al.*, 2005).

The marine *Gammaproteobacteria* showed a complex pattern, but the phylogenetic distribution of the pathways was consistent within one genus, and if available, in majority congruent with fully-sequenced species to date. They had either the *luxS* gene (*Alishewanella, Alteromonas, Shewanella*, *Vibrio*), or the *sahH* gene (*Psychrobacter*) or none of them (*Glaciecola*, *Halomonas* and *Marinobacter*).

Two genera seemed to be mixed: the *Pseudoalteromonas* and *Halomonas* species had either the *luxS* gene or none of the genes. Since closely related fully sequenced species, such as two *Pseudoalteromonas* and one *Colwellia* species, also contain none of the tested genes but the *pfs* gene, the investigated marine species could have it similarly and therefore none of the species should have the *sahH* gene. It seems probable that all species of these genera contained consistently the full two-step pathway, but some species lost the *luxS* gene, as the Pfs enzyme was sufficient to detoxify SAH.

Only one inconsistency was experienced: three almost identical *Marinobacter* species did not exhibit the *sahH* gene, although the one sequenced *Marinobacter* species has it. This discrepancy might have been caused by primer mismatch due to few reference sequences of these genera for the primer design. To get more information, further study is required with genus- or order-specific primers.

The genera of *Gammaproteobacteria* have been less explored with the exception of *Vibrio* and *Shewanella*, which both have the *luxS* gene. This was the first time that the *Alishewanella*, *Alteromonas*, *Pseudoalteromonas* and *Halomonas* species had been shown to have the *luxS* gene and that thus they could produce AI-2.

There is a debate as to the advantage for bacteria of having two enzymes instead of one for the same function. Winzer suggested that it gives the bacteria a metabolic advantage, since the two-step pathway is irreversible and detoxifies all available SAH, while the one-step pathway is reversible, and therefore dependent on the removal of the SAH catabolites (Winzer *et al.*, 2003). Nevertheless, only Pfs is crucial to detoxify SAH, LuxS appears to

be additional. Therefore, Schauder suggested that bacteria with Pfs/LuxS have the advantage of being able to produce AI-2 (Schauder *et al.*, 2001b).

5.3 Production and removal of AI-2 in the genus *Shewanella*

In all of the tested *Shewanella* species, the *luxS* gene was identified independently of the divergence of their inhabited niches. However, it has never been shown if *Shewanella* species produce AI-2, and if their AI-2 production has a correlation with the life mode.

5.3.1 AI-2 level

All *Shewanella* species produced AI-2, which was detected in their culture supernatants using the *V. harveyi* bioassay. They exhibited different maximum levels of AI-2 activity (AI-2 level): 4 to 46 % of the positive control. The pathogenic and fish-associated species (e.g. *Sh. algae*, *Sh. marinintestina*) did not show higher AI-2 level than the free-living ones (*Sh. frigidimarina*). Two species were cultivated in a protein-rich and in a marine medium, but they did not exhibit substantially different AI-2 levels. Therefore neither their life mode nor the tested cultivation conditions were connected with the AI-2 level.

As in the *Shewanella* species, also in the *Vibrio* species the life mode does not correlate with the AI-2 level (Bassler *et al.*, 1997). For example, although *V. cholerae*, *V. anguillarum* and *V. furnissii* are all pathogenic species of the genus *Vibrio*, *V. cholerae*, produced 60-120 %, while *V. anguillarum* as much as 281 % and *V. furnissii* 4 % of the AI-2 activity of *V. harveyi*.

In many studies, the AI-2 production of a species was not normalized by the positive control, but shown as fold change/ AI-2 activity (3.6.1 The *Vibrio harveyi* bioassay). Using the published values of fold changes of the tested strain and of the positive control, the relative AI-2 activities of other species were estimated and compared to *Sh. oneidensis*.

Some *Shewanella* species exhibited an AI-2 level of 10 to 50 %, analogously to *Aggregatibacter actinomycetemcomitans* (Fong *et al.*, 2001), another non-entero *Gammaproteobacteria*, and to *Eikenella corrodens*, a *Betaproteobacterium* (Azakami *et al.*, 2006), which produced approximately 20 % and 37 % of AI-2 activity of *V. harveyi* respectively. Several *Shewanella* species had an AI-2 level of under 10 %, similar to the estimated AI-2 level of *Actinomyces naeslundii*, an *Actinobacteria*, and *Streptococcus oralis*, a *Firmicutes* (Rickard *et al.*, 2006). Nevertheless, the *Shewanella* species exhibited a much lower AI-2 level than *E. coli* and *S. typhimurium* producing 100 and 237 % AI-2

activities respectively. These high AI-2 levels could be caused by the specific cultivation conditions favouring the growth of these bacteria (Surette & Bassler, 1998).

However, considering that culture supernatants were added to *V. harveyi*, the possibility can not be excluded, that some *Shewanella* species exhibited less AI-2 activity because they produced inhibiting compounds simultaneously to AI-2, as observed previously with other species e.g. *Streptococcus mutans* UA159 (Sztajer *et al.*, 2008). It is also conceivable that some *Shewanella* species converted AI-2 into other molecules or shifted the equilibrium of DPD derivatives to R-THMF, which is not detected by *V. harveyi*. This problems together with the high variability make the *V. harveyi* bioassay unreliable for quantitative analyses.

5.3.2 The pattern of AI-2 production

The *Shewanella* species exhibited a very similar pattern of AI-2 activity during growth (AI-2 pattern). Their AI-2 activity was maximal at the end of the exponential phase, and decreased in the early stationary phase. Therefore, extracellular AI-2 activity did not reflect the increasing cell-density.

This observation concurs with studies of very diverse species, like the *Bacteroidetes* species, *Porphyromonas gingivalis* (Burgess *et al.*, 2002), the *Gammaproteobacteria*, *Serratia marcescens* (Coulthurst *et al.*, 2004), and the *Firmicutes*, *Streptococcus oralis* (Rickard *et al.*, 2006).

By contrast, *V. harveyi* cultivated in AB medium did not remove AI-2 from its environment. This result is consistent with another study (Fong *et al.*, 2001), in which high (1200-fold) fold induction was measured in the overnight culture supernatant of *V. harveyi* grown in AB. It would suggest that *V. harveyi* senses AI-2, but does not remove it from its environment.

However, this is not the case under all conditions. Namely, *V. harveyi* cultivated in a heart infusion medium was shown to remove AI-2 activity, similarly to *V. vulnificus* grown in the same medium (Kim *et al.*, 2003), indicating that *V. harveyi* is as able to remove AI-2 like other bacteria. One possible explanation is that AB medium contains a component not present in the heart infusion medium, and this component persists during growth and inhibits the AI-2 removal. A similar phenomenon was observed with *S. typhimurium*, an organism which exhibited high AI-2 activity also in the stationary phase in a glucose-rich environment. Nevertheless, *S. typhimurium* still removed AI-2 after one day's incubation independently of the presence of glucose (Surette & Bassler, 1999). The other explanation

may be that *V. harveyi* secretes compounds in a heart infusion medium but not in an AB medium, compounds which degrade or convert AI-2 and thereby decrease the AI-2 activity in the heart infusion medium.

5.3.3 AI-2 synthesis and depletion

To reveal the reason, why the *Shewanella* species could exhibit this AI-2 pattern, AI-2 synthesis and depletion will be discussed.

AI-2 synthesis.

The regulation of AI-2 synthesis was thoroughly investigated in *S. typhimurium* (Beeston & Surette, 2002). In this species, the *luxS* gene was constitutively expressed, and the LuxS protein was also present in the stationary phase. Furthermore, in *trans* overexpressed *luxS* gene did not increase the AI-2 level, suggesting the expression level of LuxS is insignificant, since substrate availability determines the AI-2 level. In contrast, the expression of the *pfs* gene varied during growth, a variation which correlated with AI-2 activity. In addition the *pfs* gene was induced by the presence of carbohydrates, as observed for AI-2 activity.

Also in other species, the *luxS* gene is constitutively expressed. In *Porphyromonas gingivalis*, the LuxS protein was present during the whole growth (Burgess *et al.*, 2002) and also in *Streptococcus mutans*, the *luxS* gene was shown to be constitutively expressed (Sztajer *et al.*, 2008).

These organisms represent non-related species, and therefore it is possible that not only the genes for AI-2 synthesis but also the regulation of AI-2 synthesis is analogous in a wide range of species, potentially also in the *Shewanella* species. In conclusion, the *pfs* gene is maximally expressed at the end of the exponential phase, while the *luxS* gene is constitutively expressed, resulting in an accumulation of AI-2 in the *Shewanella* cultures in the exponential phase.

AI-2 depletion.

AI-2 depletion can be caused by two processes: uptake or degradation. In *S. typhimurium* and in *E. coli*, an uptake system via an ABC-transporter was elucidated, nevertheless in *Sh. oneidensis*, no analogous proteins can be found (Taga *et al.*, 2001; Taga *et al.*, 2003). Proteins homologous to the components of the ABC-transporter exhibit only approximately 25 % identity (data not shown). Therefore, lacking any available knowledge, AI-2 depletion was investigated in *Sh. oneidensis* by adding synthetic DPD to

Sh. oneidensis cultures, cells and culture supernatants (4.4.4.2 AI-2 depletion by the *Sh. oneidensis* strains).

AI-2 was depleted first at the end of the exponential phase independently of the extracellular AI-2 level, suggesting that the growth phase determines AI-2 depletion. Therefore, if AI-2 regulates quorum sensing-related processes either extra- or intracellularly, it should be also determined by the growth phase.

AI-2 was depleted also in the *luxS* mutant, and thus it is not induced by AI-2. In contrast, AI-2 removal of the *S. typhimurium* and the *E. coli* is induced by AI-2, and the ABC-transporter is also specific to AI-2. Since AI-2 decrease in *Sh. oneidensis* is expressed independently of AI-2, it could be a general uptake mechanism, which is not specific for it.

To elucidate if AI-2 depletion is caused by uptake or degradation, heat-killed/-inactivated and untreated cells and culture supernatants were tested for AI-2 depletion. AI-2 removal was observed in the living, but not in the dead cells therefore *Sh. oneidensis* cells take up AI-2. Interestingly, AI-2 depletion was also observed both in the non-treated and also in the heat-inactivated culture supernatants. Therefore, *Sh. oneidensis* must secrete some non-enzymatic substances that decompose or convert AI-2 into a different molecule. The exact mechanisms for the uptake and the chemical decrease remain to be elucidated.

5.4 Construction and characterization of the *Sh. oneidensis luxS* mutant

To investigate the role of AI-2 in *Sh. oneidensis*, the *luxS* gene responsible for AI-2 synthesis was knocked out in *Sh. oneidensis* and subsequently the *luxS* mutant phenotype was characterized.

Sh. oneidensis luxS mutants were constructed by two different methods. As a result of the first method, an insertional mutant and a control strain were completed, named as $luxS^-_{ins}$ and WT_{Km}. These strains had the pKnock-Km plasmid inserted in their genome: the $luxS^-_{ins}$ inserted it in the middle of the *luxS* gene, the WT_{Km} strain before the *luxS* gene in the intergenic region. As a result of the second method, a deletion *luxS* mutant without antibiotic marker ($luxS^-_{del}$) was constructed.

The $luxS^-$ insertion mutant ($luxS^-_{ins}$) and its control (WT_{Km}) were compared in terms of growth, AI-2 production, expression of secretory proteins, biofilm formation and siderophore production.

5.4.1 Construction of the *luxS* mutants

The insertional mutant.

Since this mutant had a plasmid insertion coding for kanamycin resistance and a duplication of the internal fragment of the *luxS* gene, the mutation needed to be maintained by a constant kanamycin supply. Since kanamycin could influence the phenotype independently from the mutation, a control strain was constructed. This control strain had the same plasmid insertion, but at a neutral position, in the intergenic region upstream of the *luxS* gene. This control strain is better for phenotypic comparisons than a parental strain of the *luxS* mutant, since it should also exhibit the potential adverse effects due to the kanamycin selection. This was the first time that such a control strain had been constructed, since insertion mutants of *Sh. oneidensis* have been compared only to the parental strain, which was the wildtype strain, MR-1 (De Windt *et al.*, 2006), or the rifampicin resistant strain, DSP-10 (Brown *et al.*, 2006;Thompson *et al.*, 2002).

Verifying the insertional mutant by PCR, it was noticed that a small proportion of each colony reverted to the original genome organization. Although the colonies were purified through several passages and the antibiotic selection was increased, these revertants were still detected by PCR. A similar experience was reported by other researchers as well, (Claribel Cruz-García and Dorothea Thompson, personal communication). Possibly, some revertants stuck together with the mutant or they had spontaneously developed kanamycin resistance and did not need the plasmid insertion.

The deletion mutant.

A deletion mutant without antibiotic marker was constructed using a two-step strategy: allelic replacement of *luxS* by the Gm-GFP cassette, and then excision of the cassette using the FLP recombinase. This is a well-established strategy in *Pseudomonas aeruginosa* (Boes *et al.*, 2006; Hoang *et al.*, 2000), which was adapted to *Sh. oneidensis* with some modifications.

Firstly, the source of the suicide plasmids was changed from pEX18Ap to pKnock-Km, because the plasmids with ColE1 ori, such as pEX18 plasmids, can replicate in *Sh. oneidensis*. Secondly, a kanamycin resistance marker had to be inserted into the pFLP2 plasmid, since *Sh. oneidensis* is naturally resistant to commercial β-lactam antibiotics according to experimental experiences due to the gene encoding oxacillinase on the genome (Poirel *et al.*, 2004). This was the first time that the FLP-FRT recombination system had been adapted to *Sh. oneidensis*. Alternatively, the *cre-lox* system could have been applied, as in a similar study on *Sh. oneidensis* (Cruz-Garcia *et al.*, 2007). The Cre

recombinase is also a broad-host-range recombinase as FLP, and able to remove DNA fragments flanked by the *loxP* recognition sites from genomes. For this recombinase, the specifically designed removable marker is a kanamycin resistance cassette (Marx & Lidstrom, 2002).

This deletion mutant is not an in frame mutant, but this was not necessary, since *luxS* does not form one operon with the flanking genes. To achieve in frame deletion mutants in *Sh. oneidensis*, numerous researchers (Gao *et al.*, 2006; Groh *et al.*, 2007; Wan *et al.*, 2004) adapted the method of Link and co-worker (Link *et al.*, 1997). In this method, the flanking genes are amplified by PCR and annealed together in a subsequent PCR by the tags of the primer used previously.

Allelic replacement was carried out in two sequential steps of homologous recombination, from which the second step was controlled by the *sacB* counter selection. Therefore, the *sacB* gene was additionally cloned into the allelic replacement plasmid, pBA1147. Although some contemporary researchers have done allelic replacement without s*acB* (Cruz-Garcia *et al.*, 2007; Myers & Myers, 2001), the second recombination is a very rare event, as already known for ***P. aeruginosa*** (Nellie Bös, personal communication) and experienced with *Sh. oneidensis* during this study, and therefore the use of *sacB* selection is more reliable.

Conjugation.

To introduce the suicide plasmids into *Sh. oneidensis*, conjugation was used, which is a common technique applied for plasmid introduction into *Sh. oneidensis* (Cruz-Garcia *et al.*, 2007; Myers & Myers, 2001). At first, conjugation via biparental mating was used with the multifunctional *E. coli* S17λ-*pir* strain, but this strain modified the allelic replacement plasmid. As a consequence, triparental mating was used, in which process the conjugative strain is *E. coli* HB101 with the plasmid pRK2013 and the donor strain is the *E. coli* strain optimally maintaining the mobilizable plasmid. Triparental mating proved to be a much more efficient method. It is possible that the conjugation pili might be higher expressed on the helper plasmid than on the genome of S17λ-*pir* and also, that the suicide plasmid might be at higher copy numbers in the donor cell.

After conjugation, the *Sh. oneidensis* recombinants have to be separated from the donor *E. coli* strains. To this end, auxotroph selection was applied against the *E. coli* strains by plating the conjugation mix on a defined minimal medium, a method which has already been used by other researchers (Myers & Myers, 2001). Although this auxotroph selection was not perfect, *E. coli* and *Sh. oneidensis* were easily distinguishable due to different

colony colours. Similar auxotroph selection has been applied by other researchers (Cruz-Garcia *et al.*, 2007; Gao *et al.*, 2008). They applied biparental mating with *E. coli* β2155 as the donor strain, which is analogous to S17λ-*pir* and requires diaminopimelic acid for growth. Nevertheless, this method is not applicable for triparental mating. Numerous researchers have used rifampicin selection against *E. coli* strains, since they used the *Sh. oneidensis* DSP-10 strain which is spontaneously resistant to rifampicin (Brown *et al.*, 2006; Gao *et al.*, 2006; Thompson *et al.*, 2002; Wan *et al.*, 2004). In this study, it was attempted to obtain rifampicin resistant *Sh. oneidensis*, nevertheless the *E. coli* strains used in this study were more resistant to rifampicin than *Sh. oneidensis*, and therefore this method was not applicable here.

5.4.2 Growth and AI-2 production

Both *luxS*$^{-}_{ins}$ and WT_{Km} strains grew at an identical growth rate in a complex LB medium. Although most of the *luxS* mutants did not exhibit growth deficiencies in complex media, similar to the *Sh. oneidensis luxS* mutant to date, this is not always the case. For example, the *luxS* mutant of *Lactobacillus rhamnosus* (Lebeer *et al.*, 2007) had reduced growth in a complex medium, and that of *Helicobacter pylori* showed growth delay in a Brucella serum broth (Osaki *et al.*, 2006). However, no growth defect suggests that the role of the LuxS enzyme is not essential for metabolism in *Sh. oneidensis* and in many other bacteria.

The WT_{Km} strain produced AI-2 in the wildtype manner, while the *luxS*$^{-}_{ins}$ strain did not produce AI-2, according to the intact and disrupted *luxS* genes respectively.

5.4.3 Secretome analyses

To determine the different expression of extracellular toxins or enzymes, the secretomes of the *luxS*$^{-}_{ins}$ and WT_{Km} strains were compared in LB medium at the early stationary phase. It was presumed that AI-2 regulated proteins had been expressed at this growth phase, when AI-2 activity was already decreasing.

Under the tested conditions, a marginal difference was detected, since a few proteins showed modest differential expression. Some of them were slightly upregulated, as proteins involved in motility, as MshA pilin and flagellin, and in metabolism, as malate dehydrogenase, transketolase and cysteine synthase, while some were downregulated, as two proteins that might confer resistance to toluene and antibiotics respectively.

The most interesting finding was the upregulation of the flagellin and the MshA pilin proteins. Flagellin and MshA pilin are constituents of the flagella and type IV pili

responsible for flagellar and twitching motility of the cell respectively, and these are common quorum sensing-controlled phenotypes. Reduced motility has already been observed also in other *luxS* mutants, e.g. in *Campylobacter jejuni* (Elvers & Park, 2002) and in *Helicobacter pylori* (Osaki *et al.*, 2006). In this latter species, flagella formation was influenced by AI-2 (Rader *et al.*, 2007), thus indicating the role of AI-2 as a quorum sensing signal in motility.

Both flagellar and twitching motility are involved in biofilm development in *Sh. oneidensis* and in other species. The *Sh. oneidensis* mutant without MshA pilin had a defect in adhesion to the glass surface, while mutants without flagella or with paralysed flagella were shown to form only unstructured, flat biofilms (Thormann *et al.*, 2004). This concurs with other *Gammaproteobacteria*. Namely, it was suggested that flagellar motility in *E. coli* and twitching motility in *P. aeruginosa* was involved in spreading on a glass surface during biofilm formation (Klausen *et al.*, 2003; Pratt & Kolter, 1998). Therefore upregulation of these genes in *Sh. oneidensis* would indicate a better glass coverage of the biofilm cells and eventually a different mature structure of the biofilms.

Three metabolic genes might have been upregulated due to the interruption of the activated methyl cycle and these effects were not necessarily due to quorum sensing via AI-2. Cysteine synthase might compensate for the lack of homocysteine, since its potential product, cysteine, is easily convertible to homocysteine. In addition there are indications, that this enzyme is linked to methylation processes in bacteria. In *Halobacillus halophilus*, the gene for cysteine synthase forms one operon with the *luxS* gene (Sewald *et al.*, 2007). In *Sh. oneidensis*, it is located in a different position but it is followed by an O-methyl-transferase (data not shown). The other two enzymes are commonly involved in carbon metabolism. Malate dehydrogenase is an enzyme of the citric acid cycle. Transketolase provides a link between glycolysis and pentose-phosphate pathway in both directions. These genes might be upregulated in order to compensate the lack of homocysteine, since this compound is important for the completion of the activated methyl cycle. Alternatively, they could connect S-ribosyl-homocysteine (SRH) into carbon metabolism, since SRH is the product of Pfs, the preceding enzyme to LuxS, and it is accumulated within the cell in the absence of the LuxS enzyme.

Two hypothetical proteins were downregulated. These were membrane associated proteins that are potentially involved in antibiotic and toluene resistance respectively. Similarly, higher antibiotic susceptibility has been reported in the *luxS* mutant of *Streptococcus anginosus*, a phenotype which could be complemented with DPD (Ahmed *et al.*, 2007). Therefore these phenotypes may also be quorum sensing controlled.

5.4.4 Biofilm growth

Biofilms of the *luxS*$^{-}_{ins}$ and WT_{Km} strains were grown in batch systems on glass slides in Petri dishes and on polystyrene surfaces in microtitre plates. The biofilms on the glass slide were monitored using microscopy and quantified with the Phlip toolbox (Mueller *et al.*, 2006). The biofilms in the microtitre dish were quantified using the crystal violet method (Stepanovic *et al.*, 2004).

On the glass slide, both the mature structure and the dynamic of the *luxS*$^{-}_{ins}$ and WT_{Km} biofilm were different. The mutant represented less differentiated, well-covering mats of loosely-bound cells and developed faster, while the control tended to gather in tight, very flat and round-shaped clusters.

On the polystyrene surface, the biomass of the *luxS* mutant biofilm tended to be higher. Namely, *luxS* mutant had higher biomass than the control strain in almost the whole experimental period, but the standard deviation of the method was too high to state statistical difference.

In conclusion, both the biofilm growth on the glass slides and on polycarbonate surfaces indicate that the *luxS* mutant in *Sh. oneidensis* formed slightly more biofilm and it was defective in its mature structure on the glass surface.

In most *luxS* mutants, also in *luxS* mutants of closely related *Gammaproteobacteria*, e.g. *Salmonella typhimurium* (Prouty *et al.*, 2002) and *Yersinia pestis* (Bobrov *et al.*, 2007), reduced biofilm formation was shown, but this was not true for the *Sh. oneidensis luxS* mutant.

However, similar to the *Sh. oneidensis luxS* mutant, slightly elevated biofilm formation was observed in *luxS* mutants of numerous other species, e.g. *Eikenella corrodens* (Azakami *et al.*, 2006), *Helicobacter pylori* (Cole *et al.*, 2004) and *Lactobacillus reuteri* (Tannock *et al.*, 2005).

Furthermore, similar to *Sh. oneidensis luxS* mutant, destructured biofilm was observed also in the *luxS* mutant of the closely related *Klebsiella pneumoniae*, though the biofilm was flatter and had a lower biomass (Balestrino *et al.*, 2005), in contrast to the *Sh. oneidensis luxS* mutant. Also, destroyed biofilm structure was shown in *luxS* mutants of several *Firmicutes* species, e.g. *Streptococcus mutans* (Merritt *et al.*, 2003) and *Streptococcus oralis* in co-culture with *Actynomyces naeslundii* (Rickard *et al.*, 2006).

Nevertheless, the *luxS* mutation is not only a potential quorum sensing but also a metabolic mutation, therefore complementation with AI-2 should restore biofilm phenotypes, in order to be able to state that AI-2 could be involved in the biofilm development of a given species. In other bacteria, complementation was successful on the mixed-species biofilm of the *S. oralis luxS* mutant and *A. naeslundii* (Rickard *et al.*, 2006), but it was unsuccessful on the biofilm of numerous other *luxS* mutants, e.g. that of *Salmonella typhimurium* (De Keersmaecker *et al.*, 2005), *Lactobacillus reuteri* (Tannock *et al.*, 2005), *Streptococcus mutans* (Sztajer *et al.*, 2008). Either the concentration or the timing of AI-2 addition could have been inappropriate, still these findings make it questionable, if AI-2 is involved in biofilm formation as an autoinducer in these cases. In the study reported here, complementation with AI-2 was attempted, nevertheless this experiment was unsuccessful. In this experiment, however, the inoculation method was changed, and thus also the non-complemented replica failed. Therefore, this complementation experiment remains to be undertaken.

As to the experimental procedure, a defined minimal medium (SDM) was used for biofilm cultivation. In this medium, the bacteria adhered to the glass surface faster (data not shown) than another, frequently applied complex minimal medium (LML) (Teal *et al.*, 2006; Thormann *et al.*, 2004). The biofilm staining via crystal violet could be changed to other potentially more sensitive methods such as the frequently applied Live/Dead staining (Frey *et al.*, 1995; Luther & Kamentsky, 1996) or the recently published spectrofluorometric assay (Burton *et al.*, 2007) or the newly patented BioFilm Ring Test combined with scanning electron microscopy (Chavant *et al.*, 2007), since crystal violet is not an optimal dye to stain Gram negative bacteria.

5.4.5 Siderophore production

Siderophore production of *luxS*$^{-}_{ins}$ and WT_{Km} strains was tested on a defined, minimal solid medium using the chromeazurol-S test.

The *luxS*$^{-}_{ins}$ strain produced more siderophores than the WT_{Km} strains in the first two days of incubation at 30 °C, a difference which disappeared the next day.

Similar changes were observed in the *luxS* mutant of *Aggregatibacter actinomycetemcomitans* (Fong *et al.*, 2003). In this mutant, genes encoding putative siderophore receptors were upregulated, and thus its siderophore production might be induced like in the *luxS* mutant of *Sh. oneidensis*. In the *Aggregatibacter actinomycetemcomitans luxS* mutant, not only the siderophore production was different, but the complete iron acquisition was also changed. The genes encoding inorganic iron

uptake and storage, and organic iron uptake were downregulated and the mutant grew poorly under aerobic, iron-limited conditions. In contrast, in the *Neisseria meningitidis luxS* mutant, AI-2 upregulated a putative siderophore receptor, and thus a *luxS* mutation might downregulate it, which is the opposite effect to that observed in *Sh. oneidensis* (Dove *et al.*, 2003). In *Porphyromonas gingivalis*, the *luxS* mutation also caused a disturbed iron acquisition, and AI-2 partially restored it, but this species is not able to produce siderophores (James *et al.*, 2006). Therefore, the *luxS* mutation seems to be differentially involved in the iron acquisition of several species.

In *Sh. oneidensis*, the *luxS* mutation seemed to upregulate siderophore production in a minimal medium. It remains to be identified whether A1-2 signalling caused this change and in which growth phase and under which conditions AI-2 might influence this process.

5.4.6 Conclusions

In this study, insertion and deletion *luxS* mutants as well as an insertion control were constructed. The insertion *luxS* mutant and control ($luxS^-_{ins}$ and WT_{Km}) were compared in their growth, AI-2 production, secretome, biofilm growth and siderophore production. Both strains grew at identical growth rates. WT_{Km} produced AI-2 at wildtype levels, while the $luxS^-_{ins}$ mutant did not. In the secretome study, marginal differences were observed in a complex LB medium at the end of the exponential phase. Motility and a few metabolism proteins were slightly upregulated, while hypothetical proteins potentially responsible for toluene and antibiotic resistance were slightly downregulated. In biofilm investigations, the $luxS^-_{ins}$ mutant exhibited a less-structured, less-compact biofilm structure than the WT_{Km} strain in a defined minimal medium, and the biofilm developed faster. In the siderophore analyses, the $luxS^-_{ins}$ mutant produced more siderophores than WT_{Km} strains on a defined minimal medium after two days incubation. To determine if these effects are caused by the absence of AI-2, they need to be restored by complementing AI-2.

6 Summary

1. *P. aeruginosa*, an important human pathogen, does not produce AI-2, but it was shown to moderately upregulate a few of its quorum sensing controlled virulence genes in the presence of AI-2 (Duan *et al.*, 2003). With the goal of elucidating the AI-2 controlled genes in *P. aeruginosa* in more depth, as a first step confirmation of these results was attempted for two of the published genes, *phzA1* and *lasB.* In *P. aeruginosa* PAO neither of these genes was induced by AI-2. Therefore, the previously published study could not be confirmed and the response of *P. aeruginosa* to AI-2 was not investigated thereafter.

2. AI-2 is the side product of the LuxS enzyme, which has an important role for detoxification of S-adenosyl-homocysteine (SAH) in the activated methyl cycle, and is only present in bacteria. Some bacteria, however, use an alternative enzyme for SAH recycling, the SAH hydrolase. In this study, 164 phylogenetically characterized marine isolates were investigated for the presence of the *luxS* and *sahH* genes using PCR with specific, degenerated primers and sequencing. Within one phylogenetic group, phylum or genus, these isolates used one pathway consistently. All *Alphaproteobacteria* (71) and *Bacteroidetes* (29) species contained the *sahH* gene. The *Gammaproteobacteria* (64) species exhibited a complex pattern divided by genus. *Alishewanella, Alteromonas, Shewanella* and *Vibrio* species and some *Pseudoalteromonas* and *Halomonas* species had the *luxS* gene, while the *Psychrobacter* species had the *sahH* gene. A number of *Gammaproteobacteria* (27) appeared to have neither of the genes. These results were consistent with fully-sequenced organisms, and indicate that the presence of these genes and thereby AI-2 production are determined by the phylogenetic position and biased only slightly by the life mode of a species.

3. All *Shewanella* species contained the *luxS* gene, and thus nine species of *Shewanella* as well as *Alishewanella fetalis* were investigated for AI-2 production. The maximum level of their AI-2 production was different, but did not correlate with the mode of life, e.g. free living or pathogenic. The pattern of AI-2 production during growth was similar in all species: it increased during the exponential phase, and then decreased to zero level during the early stationary phase.

4. *Shewanella oneidensis* is the best-studied, first sequenced, model organism in the *Shewanella* genus. Since nothing is known about quorum sensing in this organism, it was chosen to elucidate the role of AI-2, and thus *luxS* knockout mutants were constructed. First, an insertional mutant, $luxS^-_{ins}$, and an insertional control strain for the antibiotic resistance marker, WT_{Km}, were constructed by single homologous recombination of a suicide plasmid into the genome within the *luxS* gene ($luxS^-_{ins)}$ and immediately upstream of the *luxS* gene (WT_{Km}), respectively. These strains were then used for phenotypic comparisons. Second, a deletion mutant without antibiotic marker, $luxS^-_{del,}$ was constructed by double homologous recombination and by removal of the antibiotic marker using the FLP recombinase.

5. *Shewanella oneidensis* $luxS^-_{ins}$ and WT_{Km} strains grew at identical growth rates. As expected, the *luxS* mutant did not produce AI-2, while the control strain secreted AI-2, like the wildtype strain.

6. *Sh. oneidensis* wildtype, WT_{Km} and $luxS^-_{ins}$ strains were investigated for their ability to deplete AI-2 from their surrounding medium. AI-2 added to the strains after ten hours of growth was depleted by all three strains in a similar way, indicating that it is independent of the *luxS* mutation and the presence of AI-2. AI-2 added to the wildtype strain at the beginning of growth was not depleted till the end of the exponential phase, confirming that depletion is not regulated by AI-2, but it is rather a growth phase-dependent mechanism. In addition, the cells and the supernatants of the wildtype strain were tested for AI-2 depletion separately. The cells removed 99 % of AI-2 within half an hour. Heat-killed cells were inactive, indicating that this might be a membrane bound active uptake mechanism. Interestingly, AI-2 activity also declined in the cell-free supernatants, although at a slower rate, and independent of heat-inactivation. The mechanism is unknown, but enzymatic degradation can be excluded.

7. The secretome of *Shewanella oneidensis* $luxS^-_{ins}$ and WT_{Km} was compared by LC-MS at the end of the exponential growth-phase. Flagellin and MshA pilin related to flagellar and twitching motility respectively were slightly induced. In addition, metabolic genes were slightly induced indicating the metabolic disturbance of the LuxS mutant due to the lack of homocysteine or to the accumulation of S-ribosyl-homocysteine. Two proteins were downregulated, which are potentially responsible for resistance against toluene or aromatic compounds and antibiotics, respectively.

8. Biofilm development of *Shewanella oneidensis luxS*$^{-}_{ins}$ and WT_{Km} was monitored in a defined minimal medium on glass slides by fluorescence and confocal laser scanning microscopy. The biofilm of the *luxS*$^{-}_{ins}$ mutant developed faster, exhibited a less-structured, less-compact structure, and covered the surface better than that of the WT_{Km} strain. Biofilms were also grown on a plastic surface in microtitre plates and measured for biomass increase using crystal violet staining. The biofilm of the *luxS*$^{-}_{ins}$ mutant tended to have a higher biomass than the control.

9. The siderophore production of the *luxS*$^{-}_{ins}$ mutant and the WT_{Km} strain were analysed by spotting culture drops onto a solid defined minimal medium containing a colour indicator, chromeazurol-S, for iron chelators. The *luxS* mutant produced more siderophores than the control during the first two days of incubation.

7 References

1. **Abreu-Goodger, C. & Merino, E. (2005).** RibEx: a web server for locating riboswitches and other conserved bacterial regulatory elements. *Nucleic Acids Res* **33**, W690-W692.

2. **Ahmed, N. A., Petersen, F. C. & Scheie, A. A. (2007).** AI-2 quorum sensing affects antibiotic susceptibility in *Streptococcus anginosus*. *J Antimicrob Chemother* **60**, 49-53.

3. **Ahmer, B. M. (2004).** Cell-to-cell signalling in *Escherichia coli* and *Salmonella enterica. Mol Microbiol* **52**, 933-945.

4. **Alexeyev, M. F. (1999).** The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. *BioTechniques* **26**, 824-6, 828.

5. **Alexeyev, M. F., Shokolenko, I. N. & Croughan, T. P. (1995).** Improved antibiotic-resistance gene cassettes and omega elements for *Escherichia coli* vector construction and in vitro deletion/insertion mutagenesis. *Gene* **160**, 63-67.

6. **Allgaier, M., Uphoff, H., Felske, A. & Wagner-Dobler, I. (2003).**Aerobic anoxygenic photosynthesis in Roseobacter clade bacteria from diverse marine habitats. *Appl Environ Microbiol* **69**, 5051-5059.

7. **Antunes, L. C., Queiroz, F. L., Oliveira, F. E., Rodrigues, M. K., Eliane Santos, A. K., Maria Cavalcanti Pilotto, D. R. & Candida de Souza, F. M. (2005).** *Bacteroides* species produce *Vibrio harveyi* autoinducer 2-related molecules. *Anaerobe* **11**, 295-301.

8. **Azakami, H., Teramura, I., Matsunaga, T., Akimichi, H., Noiri, Y., Ebisu, S. & Kato, A. (2006).** Characterization of autoinducer 2 signal in *Eikenella corrodens* and its role in biofilm formation. *J Biosci Bioeng* **102**, 110-117.

9. **Balestrino, D., Haagensen, J. A., Rich, C. & Forestier, C. (2005).** Characterization of type 2 quorum sensing in *Klebsiella pneumoniae* and relationship with biofilm formation. *J Bacteriol* **187**, 2870-2880.

10. **Bassler, B. L. (1999).** How bacteria talk to each other: regulation of gene expression by quorum-sensing. *Curr Opin Microbiol* **2**, 582-587.

11. **Bassler, B. L., Greenberg, E. P. & Stevens, A. M. (1997).** Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J Bacteriol* **179**, 4043-4045.

12. **Bassler, B. L., Wright, M., Showalter, R. E. & Silverman, M. R. (1993).** Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Mol Microbiol* **9**, 773-786.

13. **Bassler, B. L., Wright, M. & Silverman, M. R. (1994).** Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway. *Mol Microbiol* **13**, 273-286.

14. **Beeston, A. L. & Surette, M. G. (2002).** *pfs*-dependent regulation of autoinducer 2 production in *Salmonella enterica* serovar *Typhimurium*. *J Bacteriol* **184**, 3450-3456.

15. **Bobrov, A. G., Bearden, S. W., Fetherston, J. D., Khweek, A. A., Parrish, K. D. & Perry, R. D. (2007).** Functional quorum sensing systems affect biofilm formation and protein expression in *Yersinia pestis*. *Adv Exp Med Biol* **603**, 178-191.

16. **Boes, N., Schreiber, K., Hartig, E., Jaensch, L. & Schobert, M. (2006).** The *Pseudomonas aeruginosa* universal stress protein PA4352 is essential for surviving anaerobic energy stress. *J Bacteriol* **188**, 6529-6538.

17. **Bompard-Gilles, C., Remaut, H., Villeret, V., Prange, T., Fanuel, L., Delmarcelle, M., Joris, B., Frere, J. & Van Beeumen, J. (2000).** Crystal structure of a D-aminopeptidase from *Ochrobactrum anthropi*, a new member of the 'penicillin-recognizing enzyme' family. *Structure* **8**, 971-980.

18. **Bowman, J. P., McCammon, S. A., Nichols, D. S., Skerratt, J. H., Rea, S. M., Nichols, P. D. & McMeekin, T. A. (1997).** *Shewanella gelidimarina* sp. nov. and *Shewanella frigidimarina* sp. nov., novel Antarctic species with the ability to produce eicosapentaenoic acid (20:5 omega 3) and grow anaerobically by dissimilatory Fe(III) reduction. *Int J Syst Evol Microbiol* **47**, 1040-1047.

19. **Boyer, H. W. & Roulland-Dussoix, D. (1969).** A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* **41**, 459-472.

20. **Bozal, N., Montes, M. J., Tudela, E., Jimenez, F. & Guinea, J. (2002).** *Shewanella frigidimarina* and *Shewanella livingstonensis* sp. nov. isolated from Antarctic coastal areas. *Int J Syst Evol Microbiol* **52**, 195-205.

21. **Brown, S. D., Martin, M., Deshpande, S., Seal, S., Huang, K., Alm, E., Yang, Y., Wu, L., Yan, T., Liu, X., Arkin, A., Chourey, K., Zhou, J. & Thompson, D. K. (2006).** Cellular response of *Shewanella oneidensis* to strontium stress. *Appl Environ Microbiol* **72**, 890-900.

22. **Burgess, N. A., Kirke, D. F., Williams, P., Winzer, K., Hardie, K. R., Meyers, N. L., Aduse-Opoku, J., Curtis, M. A. & Camara, M. (2002).** LuxS-dependent quorum sensing in *Porphyromonas gingivalis* modulates protease and haemagglutinin activities but is not essential for virulence. *Microbiology* **148**, 763-772.

23. **Burton, E., Yakandawala, N., LoVetri, K. & Madhyastha, M. S. (2007).** A microplate spectrofluorometric assay for bacterial biofilms. *J Ind Microbiol Biotechnol* **34**, 1-4.

24. **Chavant, P., Gaillard-Martinie, B., Talon, R., Hebraud, M. & Bernardi, T. (2007).** A new device for rapid evaluation of biofilm formation potential by bacteria. *J Microbiol Methods* **68**, 605-612.

25. **Chen, X., Schauder, S., Potier, N., Van Dorsselaer, A., Pelczer, I., Bassler, B. L. & Hughson, F. M. (2002).** Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**, 545-549.

26. **Chuanchuen, R., Narasaki, C. T. & Schweizer, H. P. (2002).** Benchtop and microcentrifuge preparation of *Pseudomonas aeruginosa* competent cells. *BioTechniques* **33**, 760, 762-760, 763.

27. **Chung, W. O., Park, Y., Lamont, R. J., McNab, R., Barbieri, B. & Demuth, D. R. (2001).** Signaling system in *Porphyromonas gingivalis* based on a LuxS protein. *J Bacteriol* **183**, 3903-3909.

28. **Cole, S. P., Harwood, J., Lee, R., She, R. & Guiney, D. G. (2004).** Characterization of monospecies biofilm formation by *Helicobacter pylori*. *J Bacteriol* **186**, 3124-3132.

29. **Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. & Lappin-Scott, H. M. (1995).** Microbial biofilms. *Annu Rev Microbiol* **49**, 711-745.

30. **Coulthurst, S. J., Kurz, C. L. & Salmond, G. P. (2004).** *luxS* mutants of *Serratia* defective in autoinducer-2-dependent 'quorum sensing' show strain-dependent impacts on virulence and production of carbapenem and prodigiosin. *Microbiology* **150**, 1901-1910.

31. **Cruz-Garcia, C., Murray, A. E., Klappenbach, J. A., Stewart, V. & Tiedje, J. M. (2007).** Respiratory nitrate ammonification by *Shewanella oneidensis* MR-1. *J Bacteriol* **189**, 656-662.

32. **Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. & Greenberg, E. P. (1998).** The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**, 295-298.

33. **De Keersmaecker, S. C., Varszegi, C., van Boxel, N., Habel, L. W., Metzger, K., Daniels, R., Marchal, K., De Vos, D. & Vanderleyden, J. (2005).** Chemical synthesis of (S)-4,5-dihydroxy-2,3-pentanedione, a bacterial signal molecule precursor, and validation of its activity in *Salmonella typhimurium*. *J Biol Chem* **280**, 19563-19568.

34. **De Windt, W., Gao, H., Kromer, W., Van Damme, P., Dick, J., Mast, J., Boon, N., Zhou, J. & Verstraete, W. (2006).** AggA is required for aggregation and increased biofilm formation of a hyper-aggregating mutant of *Shewanella oneidensis* MR-1. *Microbiology* **152**, 721-729.

35. **Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. (1980).** Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci U S A* **77**, 7347-7351.

36. **Dove, J. E., Yasukawa, K., Tinsley, C. R. & Nassif, X. (2003).** Production of the signalling molecule, autoinducer-2, by *Neisseria meningitidis:* lack of evidence for a concerted transcriptional response. *Microbiology* **149**, 1859-1869.

37. **Duan, K., Dammel, C., Stein, J., Rabin, H. & Surette, M. G. (2003).** Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. *Mol Microbiol* **50**, 1477-1491.

38. **Duan, K. & Surette, M. G. (2007).** Environmental regulation of *Pseudomonas aeruginosa* PAO1 Las and Rhl quorum-sensing systems. *J Bacteriol* **189**, 4827-4836.

39. **Eberhard, A., Burlingame, A. L., Eberhard, C., Kenyon, G. L., Nealson, K. H. & Oppenheimer, N. J. (1981).** Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry* **20**, 2444-2449.

40. **Elvers, K. T. & Park, S. F. (2002).** Quorum sensing in *Campylobacter jejuni*: detection of a *luxS* encoded signalling molecule. *Microbiology* **148**, 1475-1481.

41. **Figurski, D. H., Meyer, R. J. & Helinski, D. R. (1979).** Suppression of Co1E1 replication properties by the Inc P-1 plasmid RK2 in hybrid plasmids constructed in vitro. *J Mol Biol* **133**, 295-318.

42. **Fong, K. P., Chung, W. O., Lamont, R. J. & Demuth, D. R. (2001).** Intra- and interspecies regulation of gene expression by *Actinobacillus actionmycetem-comitans* LuxS. *Infect Immun* **69**, 7625-7634.

43. **Fong, K. P., Gao, L. & Demuth, D. R. (2003).** *luxS* and *arcB* control aerobic growth of *Actinobacillus actinomycetemcomitans* under iron limitation. *Infect Immun* **71**, 298-308.

44. **Freeman, J. A., Lilley, B. N. & Bassler, B. L. (2000).** A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi*. *Mol Microbiol* **35**, 139-149.

45. **Frey, T., Yue, S. & Haugland, R. P. (1995).** Dyes providing increased sensitivity in flow-cytometric dye-efflux assays for multidrug resistance. *Cytometry* **20**, 218-227.

46. **Frias, J., Olle, E. & Alsina, M. (2001).** Periodontal pathogens produce quorum sensing signal molecules. *Infect Immun* **69**, 3431-3434.

47. **Fuqua, C. & Greenberg, E. P. (2002).** Listening in on bacteria: acyl-homoserine lactone signalling. *Nat Rev Mol Cell Biol* **3**, 685-695.

48. **Fuqua, W. C., Winans, S. C. & Greenberg, E. P. (1994).** Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**, 269-275.

49. **Gao, H., Wang, X., Yang, Z. K., Palzkill, T. & Zhou, J. (2008).** Probing regulon of ArcA in *Shewanella oneidensis* MR-1 by integrated genomic analyses. *BMC Genomics* **9**, 42.

50. **Gao, W., Liu, Y., Giometti, C. S., Tollaksen, S. L., Khare, T., Wu, L., Klingeman, D. M., Fields, M. W. & Zhou, J. (2006).** Knock-out of SO1377 gene, which encodes the member of a conserved hypothetical bacterial protein family COG2268, results in alteration of iron metabolism, increased spontaneous mutation and hydrogen peroxide sensitivity in *Shewanella oneidensis* MR-1. *BMC Genomics* **7**, 76.

51. **Gonzalez, J. E. & Keshavan, N. D. (2006).** Messing with bacterial quorum sensing. *Microbiol Mol Biol Rev* **70**, 859-875.

52. **Gram, L. (1994).** Siderophore-Mediated Iron Sequestering by *Shewanella putrefaciens*. *Appl Environ Microbiol* **60**, 2132-2136.

53. **Gram, L., Bundvad, A., Melchiorsen, J., Johansen, C. & Fonnesbech, V. B. (1999).** Occurrence of *Shewanella algae* in Danish coastal water and effects of water temperature and culture conditions on its survival. *Appl Environ Microbiol* **65**, 3896-3900.

54. **Grant, S. G., Jessee, J., Bloom, F. R. & Hanahan, D. (1990).** Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. *Proc Natl Acad Sci U S A* **87**, 4645-4649.

55. **Greenberg, E. P., Hastings, J. W. & Ulitzur, S. (1979).** Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. 120 edn, pp. 87-91.

56. **Groh, J. L., Luo, Q., Ballard, J. D. & Krumholz, L. R. (2007).** Genes that enhance the ecological fitness of *Shewanella oneidensis* MR-1 in sediments reveal the value of antibiotic resistance. *Appl Environ Microbiol* **73**, 492-498.

57. **Hardie, K. R., Baldwin, T. & Williams, P. (2005).** Topley and Wilson's microbiology and microbial infections: systematic bacteriology. 10th edition. ASM Press.

58. **Hau, H. H. & Gralnick, J. A. (2007).** Ecology and biotechnology of the genus *Shewanella*. *Annu Rev Microbiol* **61**, 237-258.

59. **Heidelberg, J. F., Paulsen, I. T., Nelson, K. E., Gaidos, E. J., Nelson, W. C., Read, T. D., Eisen, J. A., Seshadri, R., Ward, N., Methe, B., Clayton, R. A., Meyer, T., Tsapin, A., Scott, J., Beanan, M., Brinkac, L., Daugherty, S., DeBoy, R. T., Dodson, R. J., Durkin, A. S., Haft, D. H., Kolonay, J. F., Madupu, R., Peterson, J. D., Umayam, L. A., White, O., Wolf, A. M., Vamathevan, J., Weidman, J., Impraim, M., Lee, K., Berry, K., Lee, C., Mueller, J., Khouri, H., Gill, J., Utterback, T. R., McDonald, L. A., Feldblyum, T. V., Smith, H. O., Venter, J. C., Nealson, K. H. & Fraser, C. M. (2002).** Genome sequence of the dissimilatory metal ion-reducing bacterium *Shewanella oneidensis*. *Nat Biotechnol* **20**, 1118-1123.

60. **Henke, J. M. & Bassler, B. L. (2004a).** Bacterial social engagements. *Trends Cell Biol* **14**, 648-656.

61. **Henke, J. M. & Bassler, B. L. (2004b).** Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi*. *J Bacteriol* **186**, 6902-6914.

62. **Higgins, D. A., Pomianek, M. E., Kraml, C. M., Taylor, R. K., Semmelhack, M. F. & Bassler, B. L. (2007).** The major *Vibrio cholerae* autoinducer and its role in virulence factor production. *Nature* **450**, 883-886.

63. **Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. & Schweizer, H. P. (1998).** A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**, 77-86.

64. **Hoang, T. T., Kutchma, A. J., Becher, A. & Schweizer, H. P. (2000).** Integration-proficient plasmids for *Pseudomonas aeruginosa*: Site-specific integration and use for engineering of reporter and expression strains. *Plasmid* **43**, 59-72.

65. **Holden, M. T., Ram, C. S., de Nys, R., Stead, P., Bainton, N. J., Hill, P. J., Manefield, M., Kumar, N., Labatte, M., England, D., Rice, S., Givskov, M., Salmond, G. P., Stewart, G. S., Bycroft, B. W., Kjelleberg, S. & Williams, P. (1999).** Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. *Mol Microbiol* **33**, 1254-1266.

66. **Holloway, B. W., Krishnapillai, V. & Morgan, A. F. (1979).** Chromosomal genetics of *Pseudomonas*. *Microbiol Rev* **43**, 73-102.

67. **Holt, H. M., Sogaard, P. & Gahrn-Hansen, B. (1997).** Ear infections with *Shewanella alga*: a bacteriologic, clinical and epidemiologic study of 67 cases. *Clin Microbiol Infect* **3**, 329-334.

68. **Holt, H. M., Gahrn-Hansen, B. & Bruun, B. (2005).** *Shewanella algae* and *Shewanella putrefaciens*: clinical and microbiological characteristics. *Clin Microbiol Infect* **11**, 347-352.

69. **Ivanova, E. P., Sawabe, T., Gorshkova, N. M., Svetashev, V. I., Mikhailov, V. V., Nicolau, D. V. & Christen, R. (2001).** *Shewanella japonica* sp. nov. *Int J Syst Evol Microbiol* **51**, 1027-1033.

70. **Ivanova, E. P., Sawabe, T., Hayashi, K., Gorshkova, N. M., Zhukova, N. V., Nedashkovskaya, O. I., Mikhailov, V. V., Nicolau, D. V. & Christen, R. (2003).** *Shewanella fidelis* sp. nov., isolated from sediments and sea water. *Int J Syst Evol Microbiol* **53**, 577-582.

71. **James, C. E., Hasegawa, Y., Park, Y., Yeung, V., Tribble, G. D., Kuboniwa, M., Demuth, D. R. & Lamont, R. J. (2006).** LuxS involvement in the regulation of genes coding for hemin and iron acquisition systems in *Porphyromonas gingivalis. Infect Immun* **74**, 3834-3844.

72. **Kaper, J. B. & Sperandio, V. (2005).** Bacterial cell-to-cell signaling in the gastrointestinal tract. *Infect Immun* **73**, 3197-3209.

73. **Khashe, S. & Janda, J. M. (1998).** Biochemical and pathogenic properties of *Shewanella alga* and *Shewanella putrefaciens*. *J Clin Microbiol* **36**, 783-787.

74. **Kim, S. Y., Lee, S. E., Kim, Y. R., Kim, C. M., Ryu, P. Y., Choy, H. E., Chung, S. S. & Rhee, J. H. (2003).** Regulation of *Vibrio vulnificus* virulence by the LuxS quorum-sensing system. *Mol Microbiol* **48**, 1647-1664.

75. **Klausen, M., Heydorn, A., Ragas, P., Lambertsen, L., Aaes-Jorgensen, A., Molin, S. & Tolker-Nielsen, T. (2003).** Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Mol Microbiol* **48**, 1511-1524.

76. **Kleerebezem, M., Quadri, L. E., Kuipers, O. P. & de Vos, W. M. (1997).** Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol Microbiol* **24**, 895-904.

77. **Kolenbrander, P. E., Andersen, R. N., Blehert, D. S., Egland, P. G., Foster, J. S. & Palmer, R. J., Jr. (2002).** Communication among oral bacteria. *Microbiol Mol Biol Rev* **66**, 486-505, table.

78. **Labrenz, M., Collins, M. D., Lawson, P. A., Tindall, B. J., Schumann, P. & Hirsch, P. (1999).** Roseovarius tolerans gen. nov., sp. nov., a budding bacterium with variable bacteriochlorophyll a production from hypersaline Ekho Lake. *Int J Syst Bacteriol* **49 Pt 1**, 137-147.

79. **Lazazzera, B. A. (2000).** Quorum sensing and starvation: signals for entry into stationary phase. *Curr Opin Microbiol* **3**, 177-182.

80. **Lebeer, S., De Keersmaecker, S. C., Verhoeven, T. L., Fadda, A. A., Marchal, K. & Vanderleyden, J. (2007).** Functional analysis of *luxS* in the probiotic strain *Lactobacillus rhamnosus* GG reveals a central metabolic role important for growth and biofilm formation. *J Bacteriol* **189**, 860-871.

81. **Lessard, I. A. & Walsh, C. T. (1999).** VanX, a bacterial D-alanyl-D-alanine dipeptidase: resistance, immunity, or survival function? *Proc Natl Acad Sci U S A* **96**, 11028-11032.

82. **Li, J., Attila, C., Wang, L., Wood, T. K., Valdes, J. J. & Bentley, W. E. (2007).** Quorum sensing in *Escherichia coli* is signaled by AI-2/LsrR: effects on small RNA and biofilm architecture. *J Bacteriol* **189**, 6011-6020.

83. **Li, M., Villaruz, A. E., Vadyvaloo, V., Sturdevant, D. E. & Otto, M. (2008).** AI-2-dependent gene regulation in *Staphylococcus epidermidis. BMC Microbiol* **8**, 4.

84. **Lilley, B. N. & Bassler, B. L. (2000).** Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. *Mol Microbiol* **36**, 940-954.

85. **Link, A. J., Phillips, D. & Church, G. M. (1997).** Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol* **179**, 6228-6237.

86. **Loh, J., Carlson, R. W., York, W. S. & Stacey, G. (2002).** Bradyoxetin, a unique chemical signal involved in symbiotic gene regulation. *Proc Natl Acad Sci U S A* **99**, 14446-14451.

87. **Luther, E. & Kamentsky, L. A. (1996).** Resolution of mitotic cells using laser scanning cytometry. *Cytometry* **23**, 272-278.

88. **Makemson, J. C., Fulayfil, N. R., Landry, W., Van Ert, L. M., Wimpee, C. F., Widder, E. A. & Case, J. F. (1997).** *Shewanella woodyi* sp. nov., an exclusively respiratory luminous bacterium isolated from the Alboran Sea. *Int J Syst Bacteriol* **47**, 1034-1039.

89. **Marx, C. J. & Lidstrom, M. E. (2002).** Broad-host-range *cre-lox* system for antibiotic marker recycling in gram-negative bacteria. *BioTechniques* **33**, 1062-1067.

90. **Merritt, J., Qi, F., Goodman, S. D., Anderson, M. H. & Shi, W. (2003).** Mutation of *luxS* affects biofilm formation in *Streptococcus mutans*. *Infect Immun* **71**, 1972-1979.

91. **Metcalf, W. W., Jiang, W. & Wanner, B. L. (1994).** Use of the rep technique for allele replacement to construct new *Escherichia coli* hosts for maintenance of R6K gamma origin plasmids at different copy numbers. *Gene* **138**, 1-7.

92. **Miller, S. T., Xavier, K. B., Campagna, S. R., Taga, M. E., Semmelhack, M. F., Bassler, B. L. & Hughson, F. M. (2004).** *Salmonella typhimurium* recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. *Mol Cell* **15**, 677-687.

93. **Mok, K. C., Wingreen, N. S. & Bassler, B. L. (2003).** *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. *EMBO J* **22**, 870-881.

94. **Mueller, L. N., de Brouwer, J. F., Almeida, J. S., Stal, L. J. & Xavier, J. B. (2006).** Analysis of a marine phototrophic biofilm by confocal laser scanning microscopy using the new image quantification software PHLIP. *BMC Ecol* **6**, 1.

95. **Myers, C. R. & Nealson, K. H. (1988).** Bacterial Manganese Reduction and Growth with Manganese Oxid as the Sole Electron Acceptor. *Science* **240**, 1319-1321.

96. **Myers, J. M. & Myers, C. R. (2001).** Role for outer membrane cytochromes OmcA and OmcB of *Shewanella putrefaciens* MR-1 in reduction of manganese dioxide. *Appl Environ Microbiol* **67**, 260-269.

97. **Nealson, K. H., Platt, T. & Hastings, J. W. (1970).** Cellular control of the synthesis and activity of the bacterial luminescent system. *J Bacteriol* **104**, 313-322.

98. **Nogi, Y., Kato, C. & Horikoshi, K. (1998).** Taxonomic studies of deep-sea barophilic *Shewanella* strains and description of *Shewanella violacea* sp. nov. *Arch Microbiol* **170**, 331-338.

99. **Nozue, H., Hayashi, T., Hashimoto, Y., Ezaki, T., Hamasaki, K., Ohwada, K. & Terawaki, Y. (1992).** Isolation and characterization of *Shewanella alga* from human clinical specimens and emendation of the description of *S. alga* Simidu et al., 1990, 335. *Int J Syst Bacteriol* **42**, 628-634.

100. **Osaki, T., Hanawa, T., Manzoku, T., Fukuda, M., Kawakami, H., Suzuki, H., Yamaguchi, H., Yan, X., Taguchi, H., Kurata, S. & Kamiya, S. (2006).** Mutation of *luxS* affects motility and infectivity of *Helicobacter pylori* in gastric mucosa of a Mongolian gerbil model. *J Med Microbiol* **55**, 1477-1485.

101. **Pagani, L., Lang, A., Vedovelli, C., Moling, O., Rimenti, G., Pristera, R. & Mian, P. (2003).** Soft tissue infection and bacteremia caused by Shewanella putrefaciens. *J Clin Microbiol* **41**, 2240-2241.

102. **Pearson, J. P., Pesci, E. C. & Iglewski, B. H. (1997).** Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriol* **179**, 5756-5767.

103. **Pecharki, D., Petersen, F. C. & Scheie, A. A. (2008).** LuxS and expression of virulence factors in *Streptococcus intermedius*. *Oral Microbiol Immunol* **23**, 79-83.

104. **Pesci, E. C. & Iglewski, B. H. (1997).** The chain of command in *Pseudomonas* quorum sensing. *Trends Microbiol* **5**, 132-134.

105. **Pesci, E. C., Milbank, J. B., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P. & Iglewski, B. H. (1999).** Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96**, 11229-11234.

106. **Poirel, L., Heritier, C. & Nordmann, P. (2004).** Chromosome-encoded ambler class D beta-lactamase of *Shewanella oneidensis* as a progenitor of carbapenem-hydrolyzing oxacillinase. *Antimicrob Agents Chemother* **48**, 348-351.

107. **Pratt, L. A. & Kolter, R. (1998).** Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. *Mol Microbiol* **30**, 285-293.

108. **Prouty, A. M., Schwesinger, W. H. & Gunn, J. S. (2002).** Biofilm formation and interaction with the surfaces of gallstones by Salmonella spp. *Infect Immun* **70**, 2640-2649.

109. **Rader, B. A., Campagna, S. R., Semmelhack, M. F., Bassler, B. L. & Guillemin, K. (2007).** The quorum-sensing molecule autoinducer 2 regulates motility and flagellar morphogenesis in *Helicobacter pylori*. *J Bacteriol* **189**, 6109-6117.

110. **Redfield, R. J. (2002).** Is quorum sensing a side effect of diffusion sensing?. *Trends Microbiol* **10**, 365-370.

111. **Rickard, A. H., Palmer, R. J., Jr., Blehert, D. S., Campagna, S. R., Semmelhack, M. F., Egland, P. G., Bassler, B. L. & Kolenbrander, P. E. (2006).** Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. *Mol Microbiol* **60**, 1446-1456.

112. **Riedel, K., Hentzer, M., Geisenberger, O., Huber, B., Steidle, A., Wu, H., Hoiby, N., Givskov, M., Molin, S. & Eberl, L. (2001).** N-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. *Microbiology* **147**, 3249-3262.

113. **Rose, T. M., Schultz, E. R., Henikoff, J. G., Pietrokovski, S., McCallum, C. M. & Henikoff, S. (1998).** Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. *Nucl Acids Res* **26**, 1628-1635.

114. **Rowe, S. M., Miller, S. & Sorscher, E. J. (2005).** Cystic fibrosis. *N Engl J Med* **352**, 1992-2001.

115. **Salgado, H., Moreno-Hagelsieb, G., Smith, T. F. & Collado-Vides, J. (2000).** Operons in *Escherichia coli*: genomic analyses and predictions. *Proc Natl Acad Sci U S A* **97**, 6652-6657.

116. **Sambrook, J. & Maniatis, T. (1989).** *Molecular Cloning: A Laboratory Manual*. 3th edition. Cold Spring Harbor Laboratory Press.

117. **Satomi, M., Oikawa, H. & Yano, Y. (2003).** *Shewanella marinintestina* sp. nov., *Shewanella schlegeliana* sp. nov. and *Shewanella sairae* sp. nov., novel eicosapentaenoic-acid-producing marine bacteria isolated from sea-animal intestines. *Int J Syst Evol Microbiol* **53**, 491-499.

118. **Schauder, S., Shokat, K., Surette, M. G. & Bassler, B. L. (2001a).** The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. *Mol Microbiol* **41**, 463-476.

119. **Schuster, M. & Greenberg, E. P. (2007).** Early activation of quorum sensing in *Pseudomonas aeruginosa* reveals the architecture of a complex regulon. *BMC Genomics* **8**, 287.

120. **Schuster, M., Lostroh, C. P., Ogi, T. & Greenberg, E. P. (2003).** Identification, Timing, and Signal Specificity of *Pseudomonas aeruginosa* Quorum-Controlled Genes: a Transcriptome Analysis. *J Bacteriol* **185**, 2066-2079.

121. **Schwyn, B. & Neilands, J. B. (1987).** Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47-56.

122. **Semmelhack, M. F., Campagna, S. R., Federle, M. J. & Bassler, B. L. (2005).** An expeditious synthesis of DPD and boron binding studies. *Org Lett* **7**, 569-572.

123. **Sewald, X., Saum, S. H., Palm, P., Pfeiffer, F., Oesterhelt, D. & Muller, V. (2007).** Autoinducer-2-producing protein LuxS, a novel salt- and chloride-induced protein in the moderately halophilic bacterium *Halobacillus halophilus*. *Appl Environ Microbiol* **73**, 371-379.

124. **Sganga, M. W., Aksamit, R. R., Cantoni, G. L. & Bauer, C. E. (1992).** Mutational and nucleotide sequence analysis of S-adenosyl-L-homocysteine hydrolase from *Rhodobacter capsulatus*. *Proc Natl Acad Sci U S A* **89**, 6328-6332.

125. **Shao, H., Lamont, R. J. & Demuth, D. R. (2007).** Autoinducer 2 is required for biofilm growth of *Aggregatibacter (Actinobacillus) actinomycetemcomitans*. *Infect Immun* **75**, 4211-4218.

126. **Shchepin, R., Hornby, J. M., Burger, E., Niessen, T., Dussault, P. & Nickerson, K. W. (2003).** Quorum sensing in *Candida albicans*: probing farnesol's mode of action with 40 natural and synthetic farnesol analogs. *Chem Biol* **10**, 743-750.

127. **Simidu, U., Kita-Tsukamoto, K., Yasumoto, T. & Yotsu, M. (1990).** Taxonomy of four marine bacterial strains that produce tetrodotoxin. *Int J Syst Evol Microbiol* **40**, 331-336.

128. **Simon, R., Priefer, U. & Pühler, A. (1983).** A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. *Bio/Technology* **1**. 784-791.

129. **Skatssoon, J. (2004).** Sun, surf and septicaemia.

130. **Sozinova, O., Jiang, Y., Kaiser, D. & Alber, M. (2006).** A three-dimensional model of myxobacterial fruiting-body formation. *Proc Natl Acad Sci U S A* **103**, 17255-17259.

131. **Sperandio, V., Torres, A. G., Jarvis, B., Nataro, J. P. & Kaper, J. B. (2003).** Bacteria-host communication: the language of hormones. *Proc Natl Acad Sci U S A* **100**, 8951-8956.

132. **Stepanovic, S., Cirkovic, I., Ranin, L. & Svabic-Vlahovic, M. (2004).** Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Lett Appl Microbiol* **38**, 428-432.

133. **Sun, J., Daniel, R., Wagner-Dobler, I. & Zeng, A. P. (2004).** Is autoinducer-2 a universal signal for interspecies communication: a comparative genomic and phylogenetic analysis of the synthesis and signal transduction pathways. *BMC Evol Biol* **4**, 36.

134. **Surette, M. G. & Bassler, B. L. (1998).** Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. *Proc Natl Acad Sci U S A* **95**, 7046-7050.

135. **Surette, M. G. & Bassler, B. L. (1999).** Regulation of autoinducer production in *Salmonella typhimurium*. *Mol Microbiol* **31**, 585-595.

136. **Surette, M. G., Miller, M. B. & Bassler, B. L. (1999).** Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. *Proc Natl Acad Sci U S A* **96**, 1639-1644.

137. **Swift, S., Downie, J. A., Whitehead, N. A., Barnard, A. M., Salmond, G. P. & Williams, P. (2001).** Quorum sensing as a population-density-dependent determinant of bacterial physiology. *Adv Microb Physiol* **45**, 199-270.

138. **Sztajer, H., Lemme, A., Vilchez, R., Schulz, S., Geffers, R., Yip, C. Y., Levesque, C. M., Cvitkovitch, D. G. & Wagner-Dobler, I. (2008).** Autoinducer-2-regulated genes in *Streptococcus mutans* UA159 and global metabolic effect of the *luxS* mutation. *J Bacteriol* **190**, 401-415.

139. **Taga, M. E., Miller, S. T. & Bassler, B. L. (2003).** Lsr-mediated transport and processing of AI-2 in *Salmonella typhimurium*. *Mol Microbiol* **50**, 1411-1427.

140. **Taga, M. E., Semmelhack, J. L. & Bassler, B. L. (2001).** The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in *Salmonella typhimurium*. *Mol Microbiol* **42**, 777-793.

141. **Takano, E. (2006).** Gamma-butyrolactones: *Streptomyces* signalling molecules regulating antibiotic production and differentiation. *Curr Opin Microbiol* **9**, 287-294.

142. **Tannock, G. W., Ghazally, S., Walter, J., Loach, D., Brooks, H., Cook, G., Surette, M., Simmers, C., Bremer, P., Dal Bello, F. & Hertel, C. (2005).** Ecological behavior of *Lactobacillus reuteri* 100-23 is affected by mutation of the *luxS* gene. *Appl Environ Microbiol* **71**, 8419-8425.

143. **Teal, T. K., Lies, D. P., Wold, B. J. & Newman, D. K. (2006).** Spatiometabolic stratification of *Shewanella oneidensis* biofilms. *Appl Environ Microbiol* **72**, 7324-7330.

144. **Thompson, D. K., Beliaev, A. S., Giometti, C. S., Tollaksen, S. L., Khare, T., Lies, D. P., Nealson, K. H., Lim, H., Yates, J., III, Brandt, C. C., Tiedje, J. M. & Zhou, J. (2002).** Transcriptional and proteomic analysis of a ferric uptake regulator (fur) mutant of *Shewanella oneidensis*: possible involvement of fur in energy metabolism, transcriptional regulation, and oxidative stress. *Appl Environ Microbiol* **68**, 881-892.

145. **Thormann, K. M., Saville, R. M., Shukla, S., Pelletier, D. A. & Spormann, A. M. (2004).** Initial Phases of biofilm formation in *Shewanella oneidensis* MR-1. *J Bacteriol* **186**, 8096-8104.

146. **Tsai, T. H. & You, H. Y. (2006).** Necrotizing fasciitis caused by Shewanella putrefaciens in a uremic patient. *J Microbiol Immunol Infect* **39**, 516-518.

147. **Tu, K. C. & Bassler, B. L. (2007).** Multiple small RNAs act additively to integrate sensory information and control quorum sensing in *Vibrio harveyi*. *Genes Dev* **21**, 221-233.

148. **Uphoff, H. U., Felske, A., Fehr, W. & Wagner-Dobler, I. (2001).**The microbial diversity in picoplankton enrichment cultures: a molecular screening of marine isolates. *FEMS Microbiol Ecol* **35**, 249-258.

149. **Venkateswaran, K., Moser, D. P., Dollhopf, M. E., Lies, D. P., Saffarini, D. A., MacGregor, B. J., Ringelberg, D. B., White, D. C., Nishijima, M., Sano, H., Burghardt, J., Stackebrandt, E. & Nealson, K. H. (1999).** Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. *Int J Syst Bacteriol* **49 Pt 2**, 705-724.

150. **Vilchez, R., Lemme, A., Thiel, V., Schulz, S., Sztajer, H. & Wagner-Dobler, I. (2007).** Analysing traces of autoinducer-2 requires standardization of the *Vibrio harveyi* bioassay. *Anal Bioanal Chem* **387**, 489-496.

151. **Vogel, B. F., Venkateswaran, K., Christensen, H., Falsen, E., Christiansen, G. & Gram, L. (2000).** Polyphasic taxonomic approach in the description of *Alishewanella fetalis* gen. nov., sp. nov., isolated from a human foetus. *Int J Syst Evol Microbiol* **50 Pt 3**, 1133-1142.

152. **Wagner-Döbler, I., Thiel, V., Eberl, L., Allgaier, M., Bodor, A., Meyer, S., Ebner, S., Hennig, A., Pukall, R. & Schulz, S. (2005).** Discovery of Complex Mixtures of Novel Long-Chain Quorum Sensing Signals in Free-Living and Host-Associated Marine *Alphaproteobacteria*. *ChemBioChem* **6**, 2195-2206.

153. **Wan, X. F., Verberkmoes, N. C., McCue, L. A., Stanek, D., Connelly, H., Hauser, L. J., Wu, L., Liu, X., Yan, T., Leaphart, A., Hettich, R. L., Zhou, J. & Thompson, D. K. (2004).** Transcriptomic and proteomic characterization of the Fur modulon in the metal-reducing bacterium *Shewanella oneidensis*. *J Bacteriol* **186**, 8385-8400.

154. **Wang, L., Hashimoto, Y., Tsao, C. Y., Valdes, J. J. & Bentley, W. E. (2005).** Cyclic AMP (cAMP) and cAMP receptor protein influence both synthesis and uptake of extracellular autoinducer 2 in *Escherichia coli*. *J Bacteriol* **187**, 2066-2076.

155. **Wang, L. H., He, Y., Gao, Y., Wu, J. E., Dong, Y. H., He, C., Wang, S. X., Weng, L. X., Xu, J. L., Tay, L., Fang, R. X. & Zhang, L. H. (2004).** A bacterial cell-cell communication signal with cross-kingdom structural analogues. *Mol Microbiol* **51**, 903-912.

156. **Waters, C. M. & Bassler, B. L. (2005).** Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* **21**, 319-346.

157. **Whiteley, M., Lee, K. M. & Greenberg, E. P. (1999).** Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96**, 13904-13909.

158. **Winzer, K., Hardie, K. R., Burgess, N., Doherty, N., Kirke, D., Holden, M. T., Linforth, R., Cornell, K. A., Taylor, A. J., Hill, P. J. & Williams, P. (2002a).** LuxS: its role in central metabolism and the in vitro synthesis of 4-hydroxy-5-methyl-3(2H)-furanone. *Microbiology* **148**, 909-922.

159. **Winzer, K., Hardie, K. R. & Williams, P. (2002b).** Bacterial cell-to-cell communication: sorry, can't talk now - gone to lunch! *Curr Opin Microbiol* **5**, 216-222.

160. **Winzer, K., Hardie, K. R. & Williams, P. (2003).** LuxS and autoinducer-2: their contribution to quorum sensing and metabolism in bacteria. *Adv Appl Microbiol* **53**, 291-396.

161. **Xavier, K. B. & Bassler, B. L. (2005).** Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*. *J Bacteriol* **187**, 238-248.

162. **Xavier, K. B., Miller, S. T., Lu, W., Kim, J. H., Rabinowitz, J., Pelczer, I., Semmelhack, M. F. & Bassler, B. L. (2007).** Phosphorylation and processing of the quorum-sensing molecule autoinducer-2 in enteric bacteria. *ACS Chem Biol* **2**, 128-136.

Appendix

Appendix A

Table AppendixA. Summary table of identification of the *luxS* and the *sahH* genes in the marine and *Shewanella* strains.

	strain	Phylogenetic affiliation	16S rRNA (%)	*luxS*	*sahH*	seq.
		Bacteroidetes				
1	LM-17	*Aquimarina sp.*	95	-	+	
2	**LM-6**	***Aquimarina sp.***	**92**	-	+	**conf.**
3	**DT-5**	***Bacteroidetes bacterium KMM 3912***	**96**	-	+	**conf.**
4	DT-2	*Donghaeana sp.*	95	-	+	
5	LM-3	*Donghaeana sp.*	95	-	+	
6	LM-2	*Krokinobacter genikus*	97	-	+	
7	LM-12	*Krokinobacter genikus*	97	-	+	
8	LM-13	*Krokinobacter genikus*	98	-	+	
9	LM-8	*Krokinobacter sp.*	96	-	+	
10	**PIC-106**	***Leeuwenhoekella accommodimaris***	**99**	-	+	**conf.**
11	DFL-18	*Rhodovirga sp.*	95	-	+	
12	DFL-17	*Rhodovirga winogradskii*	99	-	+	
13	**DFL-19**	***Rhodovirga winogradskii***	**99**	-	+	**conf.**
14	DFL-2	*Rhodovirga winogradskii*	99	-	+	
15	DFL-26	*Rhodovirga winogradskii*	98	-	+	
16	DFL-28	*Rhodovirga winogradskii*	99	-	+	
17	DFL-29	*Rhodovirga winogradskii*	99	-	+	
18	DFL-3	*Rhodovirga winogradskii*	99	-	+	
19	DFL-34	*Rhodovirga winogradskii*	99	-	+	
20	DFL-37	*Rhodovirga winogradskii*	99	-	+	

21	DFL-39	*Rhodovirga winogradskii*	99	-	+	
22	DFL-4	*Rhodovirga winogradskii*	99	-	+	
23	**DFL-40**	***Rhodovirga winogradskii***	**99**	-	+	**conf.**
24	DFL-5	*Rhodovirga winogradskii*	99	-	+	
25	DFL-6	*Rhodovirga winogradskii*	99	-	+	
26	DFL-7	*Rhodovirga winogradskii*	99	-	+	
27	DFL-8	*Rhodovirga winogradskii*	99	-	+	
28	PIC-1	*Venteria marina*	97	-	+	
29	PIC-73	*Venteria marina*	98	-	+	
		Alphaproteobacteria				
1	**BIO-60**	***Brevundimonas subvibrioides***	**98**	-	+	**conf.**
2	DFL-12	*Dinoroseobacter shibae*	100	-	+	
3	DFL-16	*Dinoroseobacter shibae*	100	-	+	
4	DFL-27	*Dinoroseobacter shibae*	100	-	+	
5	DFL-30	*Dinoroseobacter shibae*	100	-	+	
6	DFL-31	*Dinoroseobacter shibae*	100	-	+	
7	DFL-36	*Dinoroseobacter shibae*	100	-	+	
8	DFL-38	*Dinoroseobacter shibae*	100	-	+	
9	HEL-2	*Erythrobacter citreus*	98	-	+	
10	HEL-24	*Erythrobacter citreus*	97	-	+	
11	**DFL-13**	***Hoeflea phototrophica***	**100**	-	+	**conf.**
12	**DFL-33**	***Hoeflea phototrophica***	**100**	-	+	**conf.**
13	**DFL-42**	***Hoeflea phototrophica***	**100**	-	+	**conf.**
14	**DFL-43**	***Hoeflea phototrophica***	**100**	-	+	**conf.**
15	**DFL-44**	***Hoeflea phototrophica***	**100**	-	+	**conf.**
16	**LM-15**	***Hyphomonas sp.***	**91**	-	+	**conf.**
17	HEL-10	*Jannaschia helgolandensis*	100	-	+	
18	HEL-26	*Jannaschia helgolandensis*	100	-	+	
19	HEL-43	*Jannaschia helgolandensis*	100	-	+	
20	BIO-204	*Loktanella hongkongensis*	98	-	-	

21	**DT-12**	***Loktanella salsilacus***	**99**	**-**	**+**	**conf.**
22	HEL-28	*Lutibacterium anuloederans*	99	**-**	**+**	
23	BIO-13	*Methylarcula sp.*	97	**-**	**-**	
24	BIO-24	*Methylarcula sp.*	99	**-**	**-**	
25	HEL-45	*Oceanibulbus indolifex*	100	**-**	**+**	
26	HEL-76	*Oceanibulbus indolifex*	99	**-**	**+**	
27	HEL-78	*Oceanibulbus indolifex*	99	**-**	**+**	
28	DFL-9	*Oceanicaulis alexandrii*	99	**-**	**+**	
29	DFL-15	*Oceanicaulis alexandrii*	99	**-**	**+**	
30	HEL-38	*Paracoccus alkaliphilus*	97	**-**	**+**	
31	**HEL-8**	***Paracoccus sp.***	**97**	**-**	**-**	**conf.**
32	**PIC-68**	***Roseobacter sp.***	**98**	**-**	**+**	**conf.**
33	DFL-24	*Roseovarius mucosus*	100	**-**	**+**	
34	DFL-35	*Roseovarius mucosus*	100	**-**	**+**	
35	**EL-52**	***Roseovarius tolerans***	**100**	**-**	**+**	**conf.**
36	**EL-78**	***Roseovarius tolerans***	**100**	**-**	**+**	**conf.**
37	**EL-83**	***Roseovarius tolerans***	**100**	**-**	**+**	**conf.**
38	**EL-90**	***Roseovarius tolerans***	**100**	**-**	**+**	**conf.**
39	EL-164	*Roseovarius tolerans*	100	**-**	**+**	
40	EL-171	*Roseovarius tolerans*	100	**-**	**+**	
41	EL-222	*Roseovarius tolerans*	100	**-**	**+**	
42	HEL-9	*Sphingomonas baekryungensis*	99	**-**	**+**	
43	**BIO-201**	***Sphingomonas sp.***	**99**	**-**	**+**	**conf.**
44	**BIO-202**	***Sphingomonas sp.***	**99**	**-**	**+**	**conf.**
45	**BIO-203**	***Sphingomonas sp.***	**99**	**-**	**+**	**conf.**
46	**HEL-37**	***Sphingomonas sp.***	**94**	**-**	**+**	**conf.**
47	**HEL-42**	***Sphingomonas sp.***	**99**	**-**	**+**	**conf.**
48	**BIO-18**	***Sphingopyxis sp.***	**99**	**-**	**+**	**conf.**
49	LM-7	*Staleya guttiformis*	98	**-**	**+**	
50	LM-9	*Staleya guttiformis*	97	**-**	**+**	

51	**LM-10**	***Staleya sp.***	**96**	**-**	**+**	**conf.**
52	**LM-4**	***Staleya sp.***	**96**	**-**	**+**	**conf.**
53	**DFL-11**	***Stappia marina***	**98**	**-**	**+**	**conf.**
54	**LM-11**	***Sulfitobacter brevis***	**97**	**-**	**+**	**conf.**
55	**LM-16**	***Sulfitobacter brevis***	**97**	**-**	**+**	**conf.**
56	BIO-205	*Sulfitobacter dubius*	99	**-**	**+**	
57	DFL-10	*Sulfitobacter mediterraneus*	98	**-**	**+**	
58	DFL-14	*Sulfitobacter mediterraneus*	98	**-**	**+**	
59	**BIO-11**	***Sulfitobacter pontiacus***	**99**	**-**	**+**	**conf.**
60	HEL-46	*Sulfitobacter pontiacus*	98	**-**	**+**	
61	HEL-77	*Sulfitobacter pontiacus*	98	**-**	**+**	
62	**BIO-7**	***Sulfitobacter sp***	**99**	**-**	**+**	**conf.**
63	DFL-23	*Sulfitobacter sp.*	96	**-**	**+**	
64	**DFL-41**	***Sulfitobacter sp.***	**96**	**-**	**+**	**conf.**
65	PIC-69	*Sulfitobacter sp.*	96	**-**	**+**	
66	PIC-70	*Sulfitobacter sp.*	96	**-**	**+**	
67	PIC-72	*Sulfitobacter sp.*	96	**-**	**+**	
68	PIC-74	*Sulfitobacter sp.*	96	**-**	**+**	
69	PIC-76	*Sulfitobacter sp.*	95	**-**	**-**	
70	**PIC-82**	***Sulfitobacter sp.***	**96**	**-**	**+**	**conf.**
71	PIC-88	*Thalassospira lucentensis*	97	**-**	**-**	
		Gammaproteobacteria				
	ATCC 7966	***Aeromonas hydrophila***		**+**	-	**seq**
1	HEL-48	*Alteromonas atlantica*	99	**+**	-	
2	**HEL-66**	***Alteromonas atlantica***	**98**	**+**	**-**	**conf.**
3	HEL-05	*Alteromonas distincta*	100	**+**	-	
4	**HEL-51**	***Alteromonas macleodii***	**98**	**+**	**-**	**conf.**
5	BIO-267	*Alteromonas sp.*	99	**+**	-	
6	BIO-296	*Alteromonas sp.*	100	**+**	-	
7	HEL-34	*Alteromonas sp.*	75	**+**	-	

8	**HEL-50**	***Alteromonas sp.***	**100**	**+**	**-**	**conf.**
	34H	***Colwellia psychrerythraea***		**-**	**-**	**seq**
9	BIO-261	*Glaciecola sp.*	98	**-**	**-**	
10	BIO-43	*Glaciecola sp.*	94	**-**	-	
11	HEL-74	*Halomonas meridiana*	99	**+**	-	
12	HEL-18	*Halomonas sp.*	100	**+**	-	
13	**HEL-68**	***Halomonas sp.***	**98**	**+**	**-**	**conf.**
14	PIC-155	*Halomonas sp.*	98	**-**	-	
15	PIC-162	*Halomonas sp.*	95	**-**	-	
16	PIC-164	*Halomonas sp.*	98	**-**	-	
17	PIC-87	*Halomonas sp.*	98	**-**	-	
18	HEL-19	*Halomonas variabilis*	99	**+**	-	
19	HEL-33	*Halomonas variabilis*	100	**+**	-	
20	HEL-65	*Halomonas variabilis*	96	**+**	-	
21	PIC-165	*Halomonas variabilis*	98	**-**	-	
22	PIC-166	*Halomonas variabilis*	99	**+**	-	
23	BIO-294	*Halomonas venusta*	98	**-**	-	
	L2TR	***Idiomarina loihiensis***		**-**	**+**	**seq**
24	DFL-32	*Marinobacter flavimaris*	99	**-**	-	
25	DFL-20	*Marinobacter sp.*	96	**-**	-	
26	DFL-21	*Marinobacter sp.*	97	**-**	-	
27	BIO-282	*Pseudoalteromonas agarovorans*	96	**+**	-	
	T6c	***Pseudoalteromonas atlantica***		**-**	-	**seq**
28	BIO-299	*Pseudoalteromonas atlantica*	99	**-**	-	
29	BIO-304	*Pseudoalteromonas atlantica*	100	**+**	-	
30	DT-9	*Pseudoalteromonas atlantica*	99	**-**	-	
31	HEL-13	*Pseudoalteromonas atlantica*	100	**-**	-	
32	HEL-35	*Pseudoalteromonas atlantica*	99	**+**	-	
33	PIC-2	*Pseudoalteromonas atlantica*	99	**-**	-	
34	PIC-75	*Pseudoalteromonas atlantica*	99	**-**	-	

35	DT-4	*Pseudoalteromonas elyakovii*	99	**-**	-	
36	LM-1	*Pseudoalteromonas elyakovii*	99	**-**	-	
37	LM-5	*Pseudoalteromonas elyakovii*	99	**-**	-	
	TAC125	***Pseudoalteromonas haloplanktis***		**-**	-	**seq**
38	BIO-254	*Pseudoalteromonas haloplanktis*	95	**+**	-	
39	BIO-266	*Pseudoalteromonas haloplanktis*	99	**-**	-	
40	BIO-27	*Pseudoalteromonas haloplanktis*	98	**-**	-	
41	DT-3	*Pseudoalteromonas haloplanktis*	99	**-**	-	
42	DT-8	*Pseudoalteromonas haloplanktis*	99	**-**	-	
43	HEL-36	*Pseudoalteromonas nigrifaciens*	99	**+**	**-**	
44	BIO-250	*Pseudoalteromonas sp.*	98	**+**	-	
45	BIO-258	*Pseudoalteromonas sp.*	90	**+**	-	
46	BIO-270	*Pseudoalteromonas sp.*	95	**-**	-	
47	BIO-283	*Pseudoalteromonas sp.*	91	**-**	-	
48	BIO-300	*Pseudoalteromonas sp.*	99	**+**	-	
49	BIO-307	*Pseudoalteromonas sp.*	90	**+**	-	
50	DT-6	*Pseudoalteromonas sp.*	99	**-**	-	
51	DT-7	*Pseudoalteromonas sp.*	97	**-**	-	
52	HEL-14	*Pseudoalteromonas sp.*	95	**-**	-	
53	HEL-20	*Pseudoalteromonas sp.*	99	**+**	-	
54	**HEL-40**	***Pseudoalteromonas sp.***	**96**	**+**	**-**	**conf.**
55	HEL-49	*Pseudoalteromonas sp.*	99	**+**	-	
	273-4	***Psychrobacter arcticus***		**-**	**+**	**seq**
	K5	***Psychrobacter cryohalolentis***		**-**	**+**	**seq**
56	**HEL-06**	***Psychrobacter glacincola***	**100**	**-**	**+**	**conf.**
57	HEL-32	*Psychrobacter glacincola*	98	**-**	**+**	
58	**HEL-44**	***Psychrobacter immobilis***	**97**	**-**	**+**	**conf.**
	2-40	***Saccharophagus degradans***		**-**	**+**	**seq**
59	**DT-11**	***Shewanella baltica***	**99**	**+**	**-**	**conf.**

Sh	**CCUG 30811**	***AliShewanella fetalis***		**+**	**-**	**conf.**
Sh	**DSM 9167**	***Shewanella algae***		**+**	**-**	**conf.**
	OS217	***Shewanella denitrificans***		**+**	**-**	**seq**
Sh	**LMG 20552**	***Shewanella fidelis***		**+**	**-**	**conf.**
Sh	**LMG 18921**	***Shewanella frigidimarina***		**+**	**-**	**conf.**
	NCIMB 400	***Shewanella frigidimarina***		**+**	**-**	**seq**
Sh	**LMG 19691**	***Shewanella japonica***		**+**	**-**	**conf.**
Sh	**LMG 19866**	***Shewanella livingstonensis***		**+**	**-**	**conf.**
Sh	**LMG 21403**	***Shewanella marinintestina***		**+**	**-**	**conf.**
60	**DT-1**	***Shewanella hafniensis***	**98**	**+**	**-**	**conf.**
Sh	**ATCC 700500**	***Shewanella oneidensis***		**+**	**-**	**seq**
Sh	**LMG 21408**	***Shewanella sairae***		**+**	**-**	**conf.**
Sh	**LMG 21406**	***Shewanella schlegeliana***		**+**	**-**	**conf.**
	ANA-3	***Shewanella sp.***		**+**	**-**	**seq**
61	**BIO-50**	***Shewanella sp.***	**93**	**+**	**-**	**conf.**
	MR-4	***Shewanella sp.***		**+**	**-**	**seq**
	MR-7	***Shewanella sp.***		**+**	**-**	**seq**
Sh	**LMG 19151**	***Shewanella violaceae***		**+**	**-**	**conf.**
62	**BIO-305**	***Vibrio alginolyticus***	**99**	**+**	**-**	**conf.**
	El Tor N16961	***Vibrio cholerae***		**+**	**-**	**seq**
	ES114	***Vibrio fischeri***		**+**	**-**	**seq**
63	**BIO-248**	***Vibrio parahaemolyticus***	**98**	**+**	**-**	**conf.**
64	**BIO-249**	***Vibrio parahaemolyticus***	**97**	**+**	**-**	**conf.**
	RIMD 2210633	***Vibrio parahaemolyticus***		**+**	**-**	**seq**
	CMCP6	***Vibrio vulnificus***		**+**	**-**	**seq**
	YJ016	***Vibrio vulnificus***		**+**	**-**	**seq**

conf.: amplicon confirmed by sequencing; seq: fully-sequenced strain; *Sh*.: type-strains of *Shewanella* species and *Alishewanella fetalis*

Appendix B

Sequence of *Sh. oneidensis luxS*$^{-}_{ins}$

The sequenced segments are underlined, bold and italic.

‖ the border of the engineered region

XXX 3´and 5´end of the *luxS* gene

XXX *luxS*1 homologous region

XXX kanamycin resistance cassette

XXX RP4 origin of transfer

XXX R6K origin of replication

ATGCCATTACTTGATAGCT‖TTACCGTT***GACCATACTCGGATGAATGCACCTGCCGTGCG***
TGTTGCCAAACATATGACGACCCCAAAAGGCGATGCGATTACCGTATTCGATCTGCGTTT
TTGCGCGCCAAATAAAGATATCCTGAGCGAGCGTGGCATCCACACTTTGGAACATTTGTT
TGCGGGCTTTATGCGTGACCATTTAAATGGTGATGATGTGGAAATTATTGA*GGTACCGA*
GGACGCGTCGAATTAATTCCGCTAGCTTCACGCTGCCGCAAGCACTCAGGGCGCAAGGG
CTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGG
ATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCA
GGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCAA
GCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGT
AAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATC
AAGAGACAGGATGAGGATCGTTTCGCATG*ATTGAACAAGATGGATTGCACGCAGGTTCT*
CCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTG
CTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGA
CCGACCTGTCCGGTGCCCTGAATGAACTCCAAGACGAGGCAGCGCGGCTATCGTGGCTG
GCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGG
ACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCT
GCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGC
TACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGG
AAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCC
GAACTGTTCGCCAGGCTCAAGGCGCGGATGCCCGACGGCGAGGATCTCGTCGTGACCC
ATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATC
GACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTG
ATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTAT
CGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAG
CGGGACTCTGGGGTTCGCGGAATTAATTCGACGCGTCAATTCGAGGGGGATCAATTCC
GTGATAGGTGGGCTGCCCTTCCTGGTTGGCTTGGTTTCATCAGCCATCCGCTTGCCCTC
ATCTGTTACGCCGGC***GGTAGCCGGCCAGCCTCGCAGAGCAGGATTCCCGTTGAGCACC***
GCCAGGTGCGAATAAGGGACAGTGAAGAAGGAACACCCGCTCGCGGGTGGGCCTACTT
CACCTATCCTGCCCGGCTGACGCCGTTGGATACACCAAGGAAAGTCTACACGAACCCTT
TGGCAAAATCCTGTATATCGTGCGAAAAAGGATGGATATACCGAAAAAATCGCTATAAT
GACCCCGAAGCAGGGTTATGCAGCGGAAAA*CGGAATTGATCCGGCCACGATGCGTCCG*
GCGTAGAG*GATCTGAAGATCAGCAGTTCAACCTGTTGATAGTACGTACTAAGCTCTCAT*
GTTTCACGTACTAAGCTCTCATGTTTAACGTACTAAGCTCTCATGTTTAACGAACTAAAC
CCTCATGGCTAACGTACTAAGCTCTCATGGCTAACGTACTAAGCTCTCATGTTTCACGTA
CTAAGCTCTCATGTTTGAACAATAAAATTAATATAAATCAGCAACTTAAATAGCCTCTAA
GGTTTTAAGTTTTATAAGAAAAAAAAGAATATATAAGGCTTTTAAAGCTTTTAAGGTTTA

ACGGTTGTGGACAACAAGCCAGGGATGTAACGCACTGAGAAGCCCTTAGAGCCTCTCAA AGCAATTTTGAGTGACACAGGAACACTTAACGGCTGACATGGGAATTCTTACCGTTGAC CATACTCGGATGAATGCACCTGCCGTGCGTGTTGCCAAACATATGACGACCCCAAAAGG CGATGCGATTACCGTATTCGATCTGCGTTTTGCGCGCCAAATAAAGATATCCTGAGCGA GCGTGGCATCCACACTTTGGAACATTTGTTTGCGGGCTTTATGCGTGACCATTTAAATGG TGATGATGTGGAAATTATTGA‖TATTTCTCCAATGGGCTGTCGCACTGGTTTTTATATGA GCCTGATTGGCGTGCCTACTGAACGTCAAGTAGCGGATGCGTGGCTTGCCTCAATGGAA GATGTACTGAAAGTTGTTGAACAATCTGAAATTCCTGAGCTGAACGAATATCAATGTG GCACCTATGAGATGCACTCTTTGGAGCAAGCGCAGGACATTGCACGTAATATTATCGC AGCTGGCGTGAGCGTTAACCGCAACGATGATTTAAAACTCAGTGATGAGATTCTAGGT AAACTCTAG

Appendix C

Sequence of *Sh. oneidensis luxS*ΔGm-GFP

The sequenced segments are underlined, bold and italic.

‖ the border of the engineered region

XXX extracellular solute-binding protein

XXX GFP

XXX Gm

XXX TonB

ATGCGTTATTTGGGGGGACTGTTATTTACGGTATTGTTTTTTGGTGCGACTGGCTGTAA
TCCCTCATCCCAACATGCTGAACCTATTTCTAAAGCGTCCATTACTGATGCACCTCCCG
TGAAGCAAGTGATTGAATGGCGTTTAGCCACCTCTTGGCCGAAAAATTTCCCCGGCTT
AGGTATGGCGCCAGAGCGTTTTGCAACGCTGGTTAATGATATGTCCCATGGTCAATTG
CGCATTACCGTGCACGGCGCTGGGGAATTAATGCCTGCCTTTGCGGTATTCGACGGGG
TTAGCCAAGGTAAAATTCAGATGGCCCACGCCGCTTCCTATTATTGGAAGGGCAAAAC
TCCCGCCTCACAGTTTTTTTCTTCGATTCCATTTGGAATGACAGCACAGGAAATGAACG
GCTGGTTGCATTATGGCGGCATGGCGCTGTGGGAGGAGGTTTATCGCCCCTTTGGTATT
ATTCCCTTAGCAGGGGGAAATACAGGCATGCAAATGGGCGGCTGGTTTAATAAACCCA
TCAATACGATTGCCGACTTTAAAGGGCTGAAGATCCGAATGCCAGGGCTTGGTGGAGA
GGTACTTAAGCGTGTTGGCGCCGTGCCTGTTAATA***TGGCTGGCAGAGAACTGTTTAGTG***
CGCTACA‖AACAGGCTCGCTTGACGCCGCCGAATGGGTTGGGCCAGTTAACGATCTCGC
CTTTGGTTTGCATAAAGTCGCGAAATATTATTACTATCCCGGTTGGCATGAACCTGGTTC
CAATATGGAATTTTTGATCAACAAAGCCGCCTTTGAGAGTCTTCCCGAAGATTTGCAAAC
TGTCGTTAAAACAGCGGCCCGTGCTATCAATCAAGATATGTTGGATGAATACACCACGA
GCAATGTCACCGCCCTCGAAACCCTCGTTAAAGAAGAAGGTGTCGAGCTTAGGGCTTTT
CCGCCTGCGGTGTTAACCGAACTTGAGCGGATCTCACATCAGGTGATTGGAGAACAAG
CGGAACAAGATCCTATGATGGGCAAGGTGTACCGTGCTTATCATGCTTATGAACAGGG
AGTGCGTGAATACCATAAGATTTCTGAGGACGCATACAGCCAGCAACGGCAGCATTA
ATGCCATTTATTTACACAAAATGTTTATATTAG***CATCTGACTGATATGAGATGATTTCAT***
CTTAAGTTATCTTGTTATCGGTCCAAATCCATTTGATGTTATGCTTGCACCTATAAACAGA
TAGCGACAGTGGCGTTGTATCGCCCTGGGATCCCCCGGGGGTACCGAGCTCGAATTGGG
GATCTTGAAGTACCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAGAGCG
CTTTTGAAGCTGATGTGCTTAAAAACTTACTCAATGGAATTGGTCTGGACATT*TATTTGT*
ATAGTTCATCCATGCCATGTGTAATCCCAGCAGCTGTTACAAACTCAAGAAGGACCATGT
GGTCTCTCTTTTCGTTGGGATCTTTCGAAAGGGCAGATTGTGTGGACAGGTAATGGTTGT
CTGGTAAAAGGACAGGGCCATCGCCAATTGGAGTATTTTGTTGATAATGGTCTGCTAGTT
GAACGCTTCCATCTTCAATGTTGTGTCTAATTTTGAAGTTAACTTTGATTCCATTCTTTTT
GTTTGTCTGCCATGATGTATACATTGTGTGAGTTATAGTTGTATTCCAATTTGTGTCCA
AGAATGTTTCCATCTTCTTTAAAATCAATACCTTTTAACTCGATTTCTATTAACAAGGG
TATCACCTTCAAACTTGACTTTCAGCACGTGTCTTGTAGTTCCCGTCATCTTTGAAAAA
TATAGTTCTTTCCTGTACATAACCTTCGGGCATGGCACTCTTGAAAAAAGTCATGCTGT
TTCATATGATCTGGGTATCTTGAAAAGCATTGAACACCATAAGTCAGAGTAGTGACAA
GTGTTGGCCATGGAACAGGTAGTTTTCCAGTAGTGCAAATAAATTTAAGGGTAAGTTT
TCCGTATGTTGCATCACCTTCACCCTCTCCACTGACAGAAAATTTGTGCCCATTAACAT
CACCATCTAATTCAACAAGAATTGGGACAACTCCAGTGAAAAGTTCTTCTCCTTTACTC
ATATGTATATCTCCTTCTTAAATCTAGAATTCTAGATCGAATTGGCCGCGGCGTTGTGA

CAATTTACCGAACAACTCCGCGGCCGGGAAGCCGATCTCGGCTTGAACGAATTGTTAG
GTGGCGGTACTTGGGTCGATATCAAAGTGCATCACTTCTTCCCGTATGCCCAACTTTGT
ATAGAGAGCCACTGCGGGATCGTCACCGTAATCTGCTTGCACGTAGATCACATAAGCA
CCAAGCGCGTTGGCCTCATGCTTGAGGAGATTGATGAGCGCGGTGGCAATGCCCTGCC
TCCGGTGCTCGCCGGAGACTGCGAGATCATAGATATAGATCTCACTACGCGGCTGCTC
AAACTTGGGCAGAACGTAAGCCGCGAGAGCGCCAACAACCGCTTCTTGGTCGAAGGC
AGCAAGCGCGATGAATGTCTTACTACGGAGCAAGTTCCCGAGGTAATCGGAGTCCGGC
TGATGTTGGGAGTAGGTGGCTACGTCTCCGAACTCACGACCGAAAAGATCAAGAGCA
GCCCGCATGGATTTGACTTGGTCAGG***GCCGAGCCTACATGTGCGAATGATGCCCATACT***
TGAGCCACCTAACTTTGTTTTAGGGCGACTGCCCTGCTGCGTAACATCGTTGCTGCTCCA
TAACAT*CAAACATCGACCCACGGCGTAACGCGCTTGCTGCTTGGATGCCCGAGGCATAG*
ACTGTACAAAAAAACAGTCATAACAAGCCATGAAAACCGCCACTGCGCCGTTACCACCG
CTGCGTTCGGTCAAGGTTCTGGACCAGTTGCGTGAGCGCATACGCTACTTGCATTACAG
TTTACGAACCGAACAGGCTTATGTCAATTCGATCCATTGCTGTTGACAAAGGGAATCAGG
GGATCTTGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAGAGC
GCTTTTGAAGCTAATTCGAGCTCGGTACCCCGGGGGATCCTAACAACAAACGCCGTACG
CTAACAAAAAGTTTTGGGAGCTTGGT*ATGTCAAGTCGCTATTTTGTCATGTCGCTGACCG*
CGCTTGCGGTTTCCTGTGTTTACGCTAATTCACTTAATGCAAATCCCCTCTATTCTCAAA
CACTTGATTTAGCGAAGCTCAATGATCAGTATCATTCTACCGATGATGCAATGCAGTT
AAGCAATCTCAATAGCCTAACGCTGCTTCGGGCTGACCATAGTGGCAATTCCGCCAAT
ATCAGTATGCGCTCAGCCACCCAAGGTGTGGCTGTGATGCAAGATAGAGTGTATTTCT
CACCTG***CGCCCTATTCGGCACCGCAATTGCAGCTATTACCCCATCTTTTGTTGCAGCAAA***
GTGTGACGACTACGCCACTGGCCAATATGGCTGTTGGTGGTCAGGGATCATTTGGCGTG
GTGGACTACCAAAGCCTTAACATTATTGAACGCTCACAATCTGGCAGTGTTCAAATCGAG
GGGACGACGGATTCAGATGTGAATGCGGGCATGCGTTGGGGCGTTAATCAAAAAGAATA
CGGTGCCTTATTTGCGGCTAATTATATTAAAGAACAAGGTGATGCGATTTTTTCAACGG
CAGCGATGCTGACAGAAAAAAAATGGATATGTTGTTTAAGGT*CAATGCTTCAAGCTTAC*
TTGGCGCCCGCAGTCCGCAGCAAACGGAATTTACCTATCAATTTATCGATGATGATAGT
TACCGTTCATTGCTTGGGATCACTGCCGCCGACTGGCAGCAGGATCCTTTGCTGCTGTA
TTCCGCTACCGCACAAGATAAACATCAAGGACGGCAACATAAATACCAGTTATCACAT
CAAGTCAACTTAGGTGATAGCAAGGTGATGAGCGACTTTTACTATCAATTCTATTCAC
AACGACTGGATCAGCTTGGCGCGTTTAATGGGCAAAATATTGGCTTGGGAACGCTTTA
CGACATTGCCGCCTTCGATCGTAATCCCAGTGCGAATGGCGCCGATGTAAATATGTTG
GTGCAAGACAATGATTACAGTGCATTCGGTGCCCAGACTCAGTCGATAAACCAATACG
GCGAACATCAAATTACCTACAGCGCTCGTTATCATACCGATAAAGCCGAGATGCGTTT
TGGTGATCAAACTGCATTATGGCAAGCTGACAGAAGTATCGTCCGTGATGAGTCTAAT
GCGGTGTTAGCCTACACCGATGATGCCACGGCGCTGACGTCGGCAATCGACTCTTTAT
GGCGTTGGCAGGGCATGCAAATCAAGTTAGGGCTTACTTACGAGCATGTTAGTGTTAA
TCGTGAAGTGAGCTTAGCTTTTGCTGGGCTTGACGAAGCGGACTTTTCTGATAGCGATT
GGATGCCGCAACTTGGTGTGCTTTATGATGCAGGCGATTGGCGTTTTAGTACCGATATA
CGCCGGGCATGGGCTGCGGCGAGTGCGGGTAATCTTACCCAAGACGCTCAGGAATCG
CTTCATTATCAAGTCAGTGCCCAATATGCGAGTGACGGCATTAAGGCTGATTTGCGGG
CTTATGTGCAAGAATTTGATAATCTTCATGTGGATTGTGATAGTTACTCCATGTGCGTC
GATTCTCGTCTGCTAACTCAAGAAAATATACCCGATGTGCTTACCTATGGGGTTGAATT
CGGCCTGGGCTATCGCTCGATGTTGGGTGAGCTTGAATTACCCTTAGAGCTAAGTTATC
AGTATCTTAGCGCTGAATATCAGACGAGCACCTGCACCGATATCCAAGGTTGCGTCCT
GGAGGGCGACAAACTGGCTTGGTTACCCGAGCACCAATTGCAATTGAGCGCGGGGAT
TGAATACGCCCAATATCAATTAAACCTTGTCGCTGCTTATCAATCTGAGCGTGATTTCA
GTCAGTATGGTAGTGAGTTGCAGCGTGTTGACGGTCAATGGCGTGCCGATATAGCGGC
AAATTACGATATTGATAAGCACCACAGGGTGTATTTTCGCCTTGAAAACCTATTCGAT
GAATCTTTAGTGACAACAGTATCAAATAGTGGAATTCGAACTGAAAATGGACGGATCA
GCTATTTAGGTTATCAATGGCGGTTCTAA

Appendix D

Sequence of *Sh. oneidensis luxS*$^{-}_{\mathbf{del}}$

The sequenced segments are underlined, bold and italic.

‖ indicates the border of the engineered region

XXX extracellular solute-binding protein

XXX TonB

ATGCGTTATTTGGGGGGACTGTTATTTACGGTATTGTTTTTTGGTGCGACTGGCTGTAA
TCCCTCATCCCAACATGCTGAACCTATTTCTAAAGCGTCCATTACTGATGCACCTCCCG
TGAAGCAAGTGATTGAATGGCGTTTAGCCACCTCTTGGCCGAAAAATTTCCCCGGCTT
AGGTATGGCGCCAGAGCGTTTTGCAACGCTGGTTAATGATATGTCCCATGGTCAATTG
CGCATTACCGTGCACGGCGCTGGGGAATTAATGCCTGCCTTTGCGGTATTCGACGGGG
TTAGCCAAGGTAAAATTCAGATGGCCCACGCCGCTTCCTATTATTGGAAGGGCAAAAC
TCCCGCCTCACAGTTTTTTTCTTCGATTCCATTTGGAATGACAGCACAGGAAATGAACG
GCTGGTTGCATTATGGCGGCATGGCGCTGTGGGAGGAGGTTTATCGCCCCTTTGGTATT
ATTCCCTTAGCAGGGGGAAATACAGGCATGCAAATGGGCGGCTGGTTTAATAAACCCA
TCAATACGATTGCCGACTTTAAAGGGCTGAAGATCCGAATGCCAGGGCTTGGTGGAGA
GGTACTTAAGCGTGTTGGCGCCGTGCCTGTTAATATG***GCTGGCAGAGAACTGTTTAGTG***
CGCTACA*‖*AACAGGCTCGCTTGACGCCGCCGAATGGGTTGGGCCAGTTAACGATCTCGC
CTTTGGTTTGCATAAAGTCGCGAAATATTATTACTATCCCGGTTGGCATGAACCTGGTTC
CAATATGGAATTTTTGATCAACAAAGCCGCCTTTGAGAGTCTTCCCGAAGATTTGCAAAC
TGTCGTTAAAACAGCGGCCCGTGCTATCAATCAAGATATGTTGGATGAATACACCACGA
GCAATGTCACCGCCCTCGAAACCCTCGTTAAAGAAGAAGGTGTCGAGCTTAGGGCTTTT
CCGCCTGCGGTGTTAACCGAACTTGAGCGGATCTCACATCAGGTGATTGGAGAACAAGC
GGAACAAGATCCTATGATGGGCAAGGTGTACCGTGCTTATCATGCTTATGAACAGGGAG
TGCGTGAATACCATAAGATTTCTGAGGACGCATACAGCCAGCAACGGCAGCATTAA*TGC*
CATTTATTTACACAAAATGTTTATATTAGCATCTGACTGATATGAGATGATTTCATCTTAA
GTTATCTTGTTATCGGTCCAAATCCATTTGATGTTATGCTTGCACCTATAAACAGATAGC
GACAGTGGCGTTGTATCGCCCTGGGATCCCCCGGGGGGTACCGAGCTCGAATTGGGGATC
TTGAAGTACCTATTCCCTAGAAAGTATAGGAACTTCAGAGCGCTTTTGAAGCTAATTCGA
GCTCGGTACCCCGGGGGATCCTAACAACAAACGCCGTACGCTAACAAAAAGTTTTGGGA
GCTTGGTATGTCAAGTCGCTATTTTGTCATGTCGCTGACCGCGCTTGCGGTTTCCTGTGT
TTACGCTAATTCACTTAATGCAAATCCCCTCTATTCTCAAACACTTGATTTAGCGAAGCTC
AATGATCAGTATCATTCTACCGATGATGCAATGCAGTTAAGCAATCTCAATAGCCTAACG
CTGCTTCGGGCTGACCATAGTGGCAATTCCGCCAATATCAGTATGCGCTCAGCCACCCA
AGGTGTGGCTGTGATGCAAGATAGAGTGTATTTCTCACCTGCGCCCTATTCGGCACCGC
AATTGCAGCTATTACCCCATCTTTTGTTGCAGCAAAGTGTGACGACTACGCCACTGGCCA
ATATGGCTGTTGGTGGTCAGGGATCATTTGGCGTGGTGGACTACCAAAGCCTTAACATT
ATTGAACGCTCACAATCTGGCAGTGTTCAAATCGAGGGGACGACGGATTCAGATGTGAA
TGCGGGCATGCGTTGGGGCGTTAATCAAAAAGAATACGGTGCCTTATTTGCGGCTAATT
ATATTAAAGAACAAGGTGATGCGATTTTTTCAACGGCAGCGATGCTGACAGAAAAAAA
ATGGATATGTTGTTTAAGGT*‖*CAATGCTTCAAGCTTACTTGGCGCCCGCAGTCCGCAGCA
AACGGAATTTACCTATCAATTTATCGATGATGATAGTTACCGTTCATTGCTTGGGATCA
CTGCCGCCGACTGGCAGCAGGATCCTTTGCTGCTGTATTCCGCTACCGCACAAGATAA
ACATCAAGGACGGCAACATAAATACCAGTTATCACATCAAGTCAACTTAGGTGATAGC
AAGGTGATGAGCGACTTTTACTATCAATTCTATTCACAACGACTGGATCAGCTTGGCG
CGTTTAATGGGCAAAATATTGGCTTGGGAACGCTTTACGACATTGCCGCCTTCGATCGT

AATCCCAGTGCGAATGGCGCCGATGTAAATATGTTGGTGCAAGACAATGATTACAGTG
CATTCGGTGCCCAGACTCAGTCGATAAACCAATACGGCGAACATCAAATTACCTACAG
CGCTCGTTATCATACCGATAAAGCCGAGATGCGTTTTGGTGATCAAACTGCATTATGG
CAAGCTGACAGAAGTATCGTCCGTGATGAGTCTAATGCGGTGTTAGCCTACACCGATG
ATGCCACGGCGCTGACGTCGGCAATCGACTCTTTATGGCGTTGGCAGGGCATGCAAAT
CAAGTTAGGGCTTACTTACGAGCATGTTAGTGTTAATCGTGAAGTGAGCTTAGCTTTTG
CTGGGCTTGACGAAGCGGACTTTTCTGATAGCGATTGGATGCCGCAACTTGGTGTGCT
TTATGATGCAGGCGATTGGCGTTTTAGTACCGATATACGCCGGGCATGGGCTGCGGCG
AGTGCGGGTAATCTTACCCAAGACGCTCAGGAATCGCTTCATTATCAAGTCAGTGCCC
AATATGCGAGTGACGGCATTAAGGCTGATTTGCGGGCTTATGTGCAAGAATTTGATAA
TCTTCATGTGGATTGTGATAGTTACTCCATGTGCGTCGATTCTCGTCTGCTAACTCAAG
AAAATATACCCGATGTGCTTACCTATGGGGTTGAATTCGGCCTGGGCTATCGCTCGAT
GTTGGGTGAGCTTGAATTACCCTTAGAGCTAAGTTATCAGTATCTTAGCGCTGAATATC
AGACGAGCACCTGCACCGATATCCAAGGTTGCGTCCTGGAGGGCGACAAACTGGCTTG
GTTACCCGAGCACCAATTGCAATTGAGCGCGGGGATTGAATACGCCCAATATCAATTA
AACCTTGTCGCTGCTTATCAATCTGAGCGTGATTTCAGTCAGTATGGTAGTGAGTTGCA
GCGTGTTGACGGTCAATGGCGTGCCGATATAGCGGCAAATTACGATATTGATAAGCAC
CACAGGGTGTATTTTCGCCTTGAAAACCTATTCGATGAATCTTTAGTGACAACAGTATC
AAATAGTGGAATTCGAACTGAAAATGGACGGATCAGCTATTTAGGTTATCAATGGCGG
TTCTAA

Danksagung

Allen, die mich in der Zeit meiner Doktorarbeit begleitet und unterstützt haben, möchte ich an dieser Stelle Dank sagen.

Als erstes gebührt mein Dank Frau Prof. Dr. Irene Wagner-Döbler für die Möglichkeit diese Doktorarbeit am HZI anfertigen zu dürfen, und für die anspruchsvolle und interessante Promotions-Thematik. Während der Durchführung und Fertigstellung der Arbeit hat Sie mir durch entscheidende Fragen, sowie durch ihre konstruktive Kritik, immer wieder neuen Anstoß gegeben und dadurch viele Fortschritte angeregt und ermöglicht.

Bei Prof. Dr. Dieter Jahn möchte ich mich für die Übernahme des Zweitgutachtens bedanken.

Prof. Dr. Ralf Mendel danke ich für die Leitung des Doktorprüfungskomitees und für die Übernahme des dritten Fachprüfers.

Für die Mitgliedschaft in meinem Thesis Komitee danke ich Frau Dr. Susanne Häußler, Frau Dr. Brigitte Kunze, Frau Dr. Helena Sztajer und Herrn Prof. Dr. Harold Drake.

Besonderer Dank gilt an Prof Dr. Harold Drake für die Einladung nach Bayreuth und für die Gastfreundschaft, die ich in seinem Labor bei der Einführung in die anaerobe Kultivierung erfahren durfte. Ebenso bedanke ich mich bei ihm für seine wertvollen Kommentare zu meiner Arbeit und für die weite Anreise aus Bayreuth zu meinem Thesis Komitee.

Meinen Kollegen und ehemaligen Kollegen Ina Buchholz, Dr. Ramiro Vilchez-Vargas, Bettina Elxnat, Jürgen Tomasch, Thomas Riedel, André Lemme, Birte Engelhart, Johannes Leonhäuser, Sandra Meyer, Matthias Funke und Erik Pollmann danke ich für die angenehme und hilfsbereite Arbeitsatmosphäre.

Bettina Elxnat möchte ich für die kooperativen Hilfestellungen zu meinen Experimenten und meiner Laborarbeit, und für das herzliche und gemütliche Plaudern morgens im Labor, danken.

Bedanken möchte ich mich auch bei Lothar Jänsch und Joseph Wissing für die Durchführung der Sekretommessung, bei Erik Pollmann für seinen Rat und seine Hilfsbereitschaft bei Klonierungsproblemen und bei Matthias Müsken und Andreas Dötsch für die freundliche Hilfestellung beim Mikroskopieren sowie bei der Auswertung der Bilder.

Max Schobert und Nelli Bös danke ich für die Einführung in das Prinzip der Herstellung eines unmarkierten Mutanten.

Mein besonderer Dank gilt meinen Eltern, Bodorné Keresztes Mária und Bodor László, sowie meiner Schwester, Bodor Kitti, und meinem Freund, Alexander Gruber, für die unermüdliche Unterstützung während dieser Jahre. Sie haben mich in allen Umbrüchen und Veränderungen gestärkt und mir Vieles möglich gemacht, das ohne ihre Hilfe nicht möglich gewesen wäre.